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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C.371			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/009873
INTERNATIONAL APPLICATION PCT/GB00/02286	INTERNATIONAL FILING DATE 12 June 2000 (12.06.00)	PRIORITY DATE CLAIMED 11 June 1999 (11.06.99)	

TITLE OF INVENTION

DNA MANIPULATION METHODS AND APPLICATIONS FOR SYNTHETIC ENZYMES

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **(unexecuted) (3 Sheets);**
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **(4 sheets) with Form PTO-1449 (1 sheet);**
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☒ A **FIRST** preliminary amendment **(10 sheets) (along with version of markings to show changes (9 sheets));**
 - ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: **PCT International Published Application (WO 00/77181 A2) (without International Search Report) (78 sheets); Corrected Version of coversheet of PCT International Published Application (with International Search Report) (9 sheets); PCT Notification of Transmittal of the International Preliminary Examination Report (Form PCT/IPA/416) (8 sheets); Certificate of First Class Mailing (1 sheet); and Return Postcard.**

(Atty. Docket No.: SHW-009US)

IN THE UNITED STATES PATENT DESIGNATED OFFICE (DO/US)
(National Phase of International Appln.: PCT/GB00/02286,
Publication No. WO 00/77181 A2)

In re the
application of: **Anand RANGANATHAN**

International Application No.: **PCT/GB00/02286**

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For: **DNA MANIPULATION METHODS AND
APPLICATIONS FOR SYNTHETIC ENZYMES**

Attorney Docket No.: **SHW-009US**

Commissioner for Patents
BOX PCT
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Preliminary to examination of the above-referenced patent application, please amend the above-titled International patent application as follows.

In the Claims:

Please amend claims 1, 4-8, 10-12, 14-16, 20-27, 30-31-, 33, 34-46 as follows:

1. (Amended) A method of assembling several DNA units in sequence in a DNA construct, which method comprises the steps of

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- a) providing each DNA unit with a restriction enzyme recognition sequence at its 5' end and with a recognition sequence for the same restriction enzyme at its 3' end that is combined with a recognition site for a DNA modification enzyme.
- b) providing a starting DNA construct having an accessible restriction site for the same or a compatible restriction enzyme and cleaving the starting DNA construct with such a restriction enzyme,
- c) inserting the desired DNA unit and bringing the ligated product into contact with a DNA modification enzyme such that the restriction site at the 3' end of the inserted DNA unit is abolished
- d) cleaving the ligated product at an accessible unmodified recognition site for the same or a compatible restriction enzyme,
- e) repeating steps c) and d) to introduce each desired DNA unit to give a DNA construct containing all the desired units in sequence.

4. (Amended) A method of assembling several DNA units in a DNA construct which method comprises the steps of

- a) providing each DNA unit with an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5' end and with an *Xba*I recognition sequence 5'GATCTAGA3' at its 3' end,
- b) providing a starting DNA construct having an accessible *Xba*I site and cleaving the starting DNA construct with *Xba*I,

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- c) inserting the desired DNA unit and using a resulting ligated product to transform a *dam*⁺ strain of *E. coli*,
- d) recovering a resulting plasmid and cleaving the plasmid at an accessible *Xba*I site with *Xba*I,
- e) repeating steps c) and d) to introduce each desired DNA unit to give a DNA construct containing all the desired units in sequence.

5. (Amended) The method of any one of claims 1 to 3, wherein the recognition sequences for the restriction enzyme and the DNA modification enzyme are created in the DNA units prior to cutting with the restriction enzyme.

6. (Amended) The method of claims 1 to 4 wherein the restriction sites are created in the fragment by means of a primer extension reaction.

7. (Amended) The method of any one of claims 1 to 4, wherein the DNA construct is an expression vector capable of facilitating expression of the protein encoded by the desired DNA units.

8. (Amended) The method of claim 3, wherein the DNA modification is removed and the restriction site re-established by replicating the ligated product in a *dam*-strain of *E. coli* by means of a suitable vector.

10. (Amended) A method of making an assembly of several DNA units in sequence which method comprises the steps of:

- a) providing a first DNA unit with an *Xba*I recognition sequence 5'TCTAGA3' at its 3' end, and cleaving the said first DNA unit with *Xba*I,

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- b) providing each other DNA unit with a *SpeI* recognition sequence 5'ACTAGT3' at its 5' end, and downstream *XbaI* recognition sequence 5'TCTAGA3' followed by a downstream *SmaI* recognition sequence 5'CCCGGG3' at its 3' end, cleaving each said other DNA unit with *SpeI* and *SmaI*, and dephosphorylating the 5' end of the cleaved DNA unit,
 - c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product and cleaving the ligated product with *XbaI*,
 - d) ligating the product from c) with a desired DNA unit from b) to form a ligated product and cleaving the ligated product with *XbaI*
 - e) repeating step d) with each other DNA unit in turn so as to assemble the DNA units in sequence.
11. (Amended) The method of claim 9 or claim 10 wherein the assembly occurs via stepwise addition of at least one DNA unit to a vector.
12. (Amended) The method of claim 9 or claim 10 wherein the said first DNA unit is attached to the solid phase for use in step c).
14. (Amended) The method of claim 9 or claim 10, wherein the recognition sequences in one or more of the DNA units are introduced by means of extension primers.
15. (Amended) The method of claim 9 or claim 10, wherein the assembly of several DNA units is inserted in to an expression vector which is used to transform a host capable of expressing the protein encoded by the vector.

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16. (Amended) The method of any one of claims 1, 4, 9, or 10, wherein one or more of the DNA units encodes a catalytic or transport protein domain.
20. (Amended) The method of claim 16 wherein one or more of the DNA units are derived from fatty acid synthesizing enzyme domain DNA sequences.
21. (Amended) The method of claim 16 wherein one or more of the DNA units encode modules comprising one or more catalytic or transport domains.
22. (Amended) A DNA construct incorporating one or more DNA assemblies encoding synthetic enzymes made by any one of the methods of claims 1, 4, 9, or 10.
23. (Amended) A synthetic enzyme encoded by one or more DNA assemblies made by the methods of anyone of claims 1, 4, 9, or 10.
24. (Amended) A plurality of host cells expressing DNA constructs encoding one or more synthetic enzymes made by any one of the methods of claims 1, 4, 9, or 10.
25. (Amended) A plurality of transformed hybrid host cells expressing one or more DNA constructs encoding synthetic enzymes incorporating a DNA assembly made by any one of the methods of claims 1, 4, 9, or 10.
26. (Amended) A compound produced by a synthetic enzyme encoded by a DNA assembly made by any one of the methods of claims 1, 4, 9, or 10.
27. (Amended) A method of synthesizing a target molecule comprising the steps of
 - a) examining the composition and stereochemistry of a target molecule,

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- b) determining which catalytic and transport domains need to be present in a synthetic enzyme in order to catalyze the synthesis of the target molecule,
- c) using any one of the methods of claims 1, 4, 9, or 10 to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes said synthetic enzyme which is capable of synthesizing the target molecule.
- d) placing the DNA assembly into a vector to allow expression of the synthetic enzyme in a host capable of synthesizing the target molecule after transformation with said vector.

30. (Amended) A method for producing a target molecule using the transformed host of claim 27.

31. (Amended) A method of making a synthetic enzyme to catalyze the synthesis of a target molecule comprising the steps of

- a) examining the composition and stereochemistry of a target molecule,
- b) determining which catalytic and transport domains need to be present in the synthetic enzyme in order to catalyze the synthesis of the target molecule.
- c) using any one of the methods of claims 1, 4, 9, or 10 to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes an enzyme which is capable of synthesizing the target molecule.
- d) expressing the DNA assembly in a suitable host to produce the enzyme.

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32. (Amended) A library of DNA units encoding catalytic or transport protein domains, wherein each DNA unit has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

33. (Amended) The library of claim 32, wherein each DNA units has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end.

35. (Amended) The library of claim 34, wherein each DNA unit has a *Spe*I recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3'-end.

36. (Amended) The library of claim 32 or claim 34, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

37. (Amended) A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

38. (Amended) The module as claimed in claim 37, wherein the module has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end.

39. (Amended) A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed a downstream recognition sequence for a third restriction enzyme at its 3' end, such that the DNA units, once restricted by the first and second restriction enzymes can be ligated together to abolish the restriction sites at the ligation junction.

40. (Amended) The module as claimed in claim 39, wherein the module has a *SpeI* recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *XbaI* recognition sequence 5'TCTAGA3' followed by a downstream *SmaI* recognition sequence 5'CCCGGG3' at its 3'-end.

41. (Amended) A module as claimed in claim 37 or claim 39, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

42. (Amended) A vector containing one or more modules as claimed in claim 37 or claim 39.

43. (Amended) The vector as claimed in claim 42, wherein a non-functional *recA* gene is also present.

44. (Amended) A method of transforming a host with one or more synthetic DNA assemblies encoding enzyme domains which comprises the steps of:

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- a) inserting said DNA assembly into a vector containing a mutated internal fragment of a *recA* gene sequence such that the vector is capable of undergoing homologous recombination with the *recA* gene of the host,
- b) bringing said vector into contact with a host chromosome under conditions which permit homologous recombination to take place,
- c) disrupting the host *recA* gene by the integration of the DNA of said vector into the chromosome.

45. The method of claim 44 wherein the expression vector is used to transform a *Streptomyces* host.

46. (Amended) The method of claim 44 or claim 45, wherein the DNA assemblies are modules according to claim 35 or claim 37.

47. (Amended) A host lacking a *recA* function, transformed with a vector containing one or more modules according to claim 35 or 37.

48. (Amended) A kit containing an element selected from the group consisting of DNA units, DNA modules, vectors, DNA manipulation hosts, DNA modification hosts, expression hosts, and solid phase elements for use in any one of the methods of claims 1, 4, 9, 10, or 44.

REMARKS

Applicant amends claims 1, 4-8, 10-12, 14-16, 20-27, 30-31-, 33, 34-46 and rennumbers claims 34-44 to 36-48, to address minor formal matters to conform the claims

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

1. (Amended) A method of assembling several DNA units in sequence in a DNA construct, which method comprises the steps of
 - a) providing each DNA unit with a restriction enzyme recognition sequence at its 5' end and with a recognition sequence for the same restriction enzyme at its 3' end that is combined with a recognition site for a DNA modification enzyme.
 - b) providing a starting DNA construct having an accessible restriction site for the same or a compatible restriction enzyme and cleaving the starting DNA construct with such a restriction enzyme,
 - c) inserting the desired DNA unit and bringing the ligated product into contact with a DNA modification enzyme such that the restriction site at the 3' end of the inserted DNA unit is abolished
 - d) cleaving the ligated product at an accessible unmodified recognition site for the same or a compatible restriction enzyme,
 - e) repeating steps c) and d) to introduce each desired DNA unit to give a DNA construct containing all the desired units in sequence.
4. (Amended) A method of assembling several DNA units in a DNA construct~~The method of claim 3~~ which method comprises the steps of

- providing each DNA unit with an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5' end and with an *Xba*I recognition sequence 5'GATCTAGA3' at its 3' end,
- providing a starting DNA construct having an accessible *Xba*I site and cleaving the starting DNA construct with *Xba*I,
- inserting the desired DNA unit and using a resulting ligated product to transform a *dam*⁺ strain of *E. coli*,
- recovering a resulting plasmid and cleaving the plasmid at an accessible *Xba*I site with *Xba*I,
- repeating steps c) and d) to introduce each desired DNA unit to give a DNA construct containing all the desired units in sequence.

5. (Amended) The method of any one of claims 1 to ~~43~~4, wherein the recognition sequences for the restriction enzyme and the DNA modification enzyme are created in the DNA units prior to cutting with the restriction enzyme.

6. (Amended) The method of claims 1 to 4~~claim 5~~ wherein the restriction sites are created in the fragment by means of a primer extension reaction.

7. (Amended) The method of any one of claims 1 to 4~~1 to 6~~, wherein the DNA construct is an expression vector capable of facilitating expression of the protein encoded by the desired DNA units.

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8. (Amended) The method of claims 3 ~~or claim 4~~, wherein the DNA modification is removed and the restriction site re-established by replicating the ligated product in a dam-strain of *E. coli* by means of a suitable vector.

10. (Amended) A method of making an assembly of several DNA units in sequence ~~The method of claim 9~~ which method comprises the steps of:

- a) providing a first DNA unit with an *Xba*I recognition sequence 5'TCTAGA3' at its 3' end, and cleaving the said first DNA unit with *Xba*I,
- b) providing each other DNA unit with a *Spe*I recognition sequence 5'ACTAGT3' at its 5' end, and downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3' end, cleaving each said other DNA unit with *Spe*I and *Sma*I, and dephosphorylating the 5' end of the cleaved DNA unit,
- c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product and cleaving the ligated product with *Xba*I,
- d) ligating the product from c) with a desired DNA unit from b) to form a ligated product and cleaving the ligated product with *Xba*I
- e) repeating step d) with each other DNA unit in turn so as to assemble the DNA units in sequence.

11. (Amended) The method of claim 9 or claim 10 wherein the assembly occurs via stepwise addition of at least one DNA unit ~~fragments~~ to a vector.

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12. (Amended) The method of claim 9 or claim 10 wherein the said first DNA unit is attached to the solid phase for use in step c).
14. (Amended) The method of claim 9 or claim 10~~any one of claims 9-13~~, wherein the recognition sequences in one or more of the DNA units are introduced by means of extension primers.
15. (Amended) The method of claim 9 or claim 10, ~~any one of claims 9-14~~ wherein the assembly of several DNA units is inserted in to an expression vector which is used to transform a host capable of expressing the protein encoded by the vector.
16. (Amended) The method of any one of claims 1, 4, 9, or 10-15, wherein one or more of the DNA units encodes a catalytic or transport protein domain. ~~(see Kleinkauf peptide/polyketide systems paper)~~
20. (Amended) The method of claim 16 wherein one or more of the DNA units are derived from fatty acid ~~synthesizing~~synthesising enzyme domain DNA sequences.
21. (Amended) The method of claim 16 wherein one or more of the DNA units encode modules comprising one or more catalytic or transport domains.
22. (Amended) A DNA ~~construct~~constructs incorporating one or more DNA assemblies encoding synthetic enzymes made by any one of the methods of claims 1, 4, 9, or 10-21.
23. (Amended) A ~~synthetic enzyme~~Synthetic enzymes encoded by one or more DNA assemblies made by the methods of anyone of claims 1, 4, 9, or 10-21.
24. (Amended) A plurality of host cells~~Hosts~~ expressing DNA constructs encoding one or more synthetic enzymes made by any one of the methods of claims 1, 4, 9, or 10-21.

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25. (Amended) ~~A plurality of transformed hybrid host cells~~ Hybrids of transformed hybrid host cells expressing one or more DNA constructs encoding synthetic enzymes incorporating a DNA assembly made by any one of the methods of claims 1, 4, 9, or 10-~~21~~.

26. (Amended) ~~A compound~~ Compounds produced by ~~a synthetic enzyme~~ enzymes encoded by ~~a DNA assembly~~ assemblies made by any one of the methods of claims 1, 4, 9, or 10-~~21~~.

27. (Amended) A method of ~~synthesizing~~ synthesising a target molecule comprising the steps of

- a) examining the composition and stereochemistry of a target molecule,
- b) determining which catalytic and transport domains need to be present in a synthetic enzyme in order to ~~catalyze~~ catalyse the synthesis of the target molecule,
- c) using any one of the methods of claims 1, 4, 9, or 10-~~21~~ to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes said synthetic enzyme which is capable of ~~synthesizing~~ synthesising the target molecule.
- d) placing the DNA assembly into a vector to allow expression of the synthetic enzyme in a host capable of ~~synthesizing~~ synthesising the target molecule after transformation with said vector.

30. (Amended) ~~A method for producing a target molecule using the~~ Use of transformed host of claim 27 ~~to produce said target molecule.~~

31. (Amended) A method of making a synthetic enzyme to catalyze~~catalyse~~ the synthesis of a target molecule comprising the steps of

- a) examining the composition and stereochemistry of a target molecule,
- b) determining which catalytic and transport domains need to be present in the synthetic enzyme in order to catalyze~~catalyse~~ the synthesis of the target molecule.
- c) using any one of the methods of claims 1, 4, 9, or 10-21 to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes an enzyme which is capable of synthesizing~~synthesis~~ the target molecule.
- d) expressing the DNA assembly in a suitable host to produce the enzyme.

32. (Amended) A library of DNA units encoding catalytic or transport protein domains, wherein each DNA unit has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

33. (Amended) The library of claim 32, wherein each DNA units has an *Xba*I recognition sequence 5'X~~XT~~CTAGA3' (where XX is not GA) at ~~its~~its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at ~~its~~its 3'-end.

35. (Amended) The library of claim 34, wherein each DNA unit has a *SpeI* recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *XbaI* recognition sequence 5'TCTAGA3' followed by a downstream *SmaI* recognition sequence 5'CCCGGG3' at its 3'-end.

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3634. (Amended) The library of claim 32 or claim 34, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

3735. (Amended) A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

3836. (Amended) The module as claimed in claim 3735, wherein the module has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end.

3937. (Amended) A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed a downstream recognition sequence for a third restriction enzyme at its 3' end, such that the DNA units, once restricted by the first and second restriction enzymes can be ligated together to abolish the restriction sites at the ligation junction.

4038. (Amended) The module as claimed in claim 3937, wherein the module has a *Spe*I recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3'-end.

4139. (Amended) A module as claimed in claim 3735 or claim 3937, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT

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domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

4240. (Amended) A vector containing one or more modules as claimed in claim 3735 or claim 3937.

4341. (Amended) The vector as claimed in claim 4240, wherein a non-functional recA gene is also present.

4442. (Amended) A method of transforming a host with one or more synthetic DNA assemblies encoding enzyme domains which comprises the steps of:

- a) ~~inserting~~Inserting said DNA assembly into a vector containing a mutated internal fragment of a recA gene sequence such that the vector is capable of undergoing homologous recombination with the recA gene of the host,
- b) bringing said vector into contact with a host chromosome under conditions which permit homologous recombination to take place,
- c) disrupting the host recA gene by the integration of the DNA of said vector into the chromosome.

4543. The method of claim 4442 wherein the expression vector is used to transform a *Streptomyces* host.

4644. (Amended) The method of claim 4442 or claim 4543, wherein the DNA assemblies are modules according to claim 35 or claim 37.

4846. (Amended) A kit containing an element selected from the group consisting of
DNA units, DNA modules, vectors, DNA manipulation hosts, DNA modification hosts,
expression hosts, and/or solid phase elements for use in any one of the methods of claims
1, 4, 9, 10, or 44the methods claimed herein.

DNA MANIPULATION METHODS AND APPLICATIONS FOR
SYNTHETIC ENZYMES.

5 Background

 Polyketides, including the valuable drugs avermectin, erythromycin and rapamycin, are natural products that are synthesised by stepwise condensation of acetate, propionate and occasionally butyrate units. The enzymes that take part in the biosynthesis of polyketide chains are collectively known as the polyketide synthase (PKS). PKSs include examples of both type I (multifunctional enzyme) and type II (dissociable complex) organisation. The sequencing of the gene clusters encoding the erythromycin- (ery) and rapamycin- (rap) producing polyketide synthases has shown that each cycle of polyketide chain extension is catalysed by a different set or 'module' of enzyme activities, housed in a few very large multienzyme polypeptides. The basic building blocks of modules are enzymatic 'domains' that are covalently linked together. The ability of these domains to act upon the carbon chain and remove/add functionalities is reminiscent of a molecule being acted upon by chemical reagents in a chemical synthesis. The aim is therefore to assemble these domains or even modules in a manner as desired, so that the linked enzymes can carry out efficient synthesis of any target molecule. Until now, it has however not been possible to find a versatile methodology to assemble these PKS units.

25 The whole area of polyketide research is at a stage where the flexibility of the whole enzymatic machinery is understood, despite the lack of any X-ray crystal structure data on these giant enzymes, but it remains difficult to "re-assemble" the enzymes *de novo*. A *de novo* synthesis is desirable for two reasons. Firstly, one does not need to change the structure of, for example, an antibiotic using tedious chemical
30 methodologies that are time-consuming and expensive. Engineering an

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synthetic enzyme at the genetic level is much easier, faster and cheaper. As more and more antibiotics are rendered useless, simply because the bacteria they were active against have developed ways in which to become resistant to these drugs, there is an urgency to keep developing altered
5 drug structures. Secondly, there is an ever-growing need for new drugs, more potent in their action than their predecessors. Whilst nature provides a large proportion of the new molecules that are, for example, antibiotic, anticholesterol, antifungal, or anti-cancer, the complicated structures of these drugs (for example the anti-cancer Taxol) makes it increasingly
10 difficult for chemists to carry out conventional syntheses. The problem is made more difficult by the fact that the genes that make these drugs cannot always be isolated.

The isolation of the genes coding for the proteins that make the highly potent anti-cancer compound Taxol, has not as yet been reported.
15 The resulting choice for obtaining Taxol is either to cut down 200 Pacific Yew trees to obtain enough taxol for one chemotherapy session, or to make the drug chemically using one of the many exceedingly expensive and long chemical routes that have appeared recently in the literature.

With the isolation, cloning and sequencing of the genes coding for
20 the erythromycin polyketide synthases, a model for the functioning of modular type I PKSs began to emerge. It was clear that such a system is genetically programmed to carry out the necessary catalytic activities needed for processing of the polyketide chain. It is hypothesised that each domain acts independently on the progressing carbon skeleton and there is
25 a correlation between the structure of the growing chain and the enzymatic activities carried out by the enzymes.

The first conclusive proof of such an arrangement came from experiments done by Donadio *et al.* (1991, 1993). One such experiment (1991) involved an in-frame deletion in the ORF3 segment of erythromycin
30 chromosome. This deletion eliminated the entire 183 amino acids of the ketoreductase domain of *ery* PKS module 5, along with some of the

flanking region (a total of 271 amino acids) and resulted in the production of 5,6-dideoxy-3- α -mycarosyl-5-oxo-erythronolide B, the structure of which was confirmed by X-ray crystallography. Replacement of two amino acids in the putative NAD(P)H-binding motif of the enoylreductase domain
5 encoded by ORF2 resulted in a new macrolide $\Delta^{6,7}$ -anhydroerythromycin C being produced albeit in low yield. These results demonstrated that erythromycin PKS can be genetically reprogrammed to produce novel macrolides that would otherwise be difficult to get via chemical means.

During the analysis of the fermentation products produced by a
10 strain of *S. erythraea* that was genetically engineered to produce an analogue of 6dEB, it was found that a minor component of the fermentation was 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ -lactone (Donadio *et al.*, 1991). This product was predicted to result from premature release of the chain from either the ACP of module 2 or the KS of module 3. A greater
15 yield of this triketide product was obtained by heterologous over-expression of ORF1 in *Streptomyces coelicolor* (Kao *et al.*, 1994), which also showed that DEBS1 can function autonomously. More recently (Cortés *et al.*, 1995), a six-membered lactone was produced through genetically engineering the PKS. By repositioning the TE (cyclase) domain from module 6 to the C-
20 terminus of module 2 (end of DEBS1), it was found that the yield of the lactone is increased by five-fold to 10-15 mg/L as compared to 1-3 mg/L obtained by Kao *et al.*

The relocation of the thioesterase domain at the end of DEBS1 was the first example demonstrating the efficacy of repositioning domains in
25 type I modular systems. Since then, numerous such experiments have been carried out in order to probe further the efficacy of these multienzymes. The TE domain has been relocated at the end of module 5 as well as module 3 respectively (Kao *et al.*, 1995, 1996). In both cases, the predicted compounds were produced that resulted from truncation of
30 the progressing polyketide chain. Release of the 12-membered product in the former case showed that the thioesterase domain can indeed catalyse

ring closure even for less energetically favourable reactions. In the second experiment, two products were produced, one of them thought to be resulting from spontaneous decarboxylation.

The first example of a chimaeric polyketide synthase constructed
5 from a domain taken from a second PKS was demonstrated by Oliynyk *et al.* (1996). An acyltransferase domain (AT) from module 2 of the rapamycin polyketide synthase was used to replace the AT of module 1 in the DEBS1-TE system. The resulting triketide lactone had a methyl group missing at position 5 of the six-membered ring. This was expected since the AT of
10 module 2 of *rap* PKS (unlike the AT of module 1 of DEBS1) incorporates a malonyl-CoA extender unit, instead of a methylmalonyl-CoA unit.

Thus, it has been shown that not only can domains residing within a particular PKS be interchanged or destroyed, analogous domains can be derived from other synthases for the same purpose or for achieving the
15 required synthetic goal. Such a strategy immediately provides a glimpse of the manner in which "designer" polyketides can be constructed through using "off-the-shelf" gene products.

More recently, another hybrid system has been constructed (Marsden *et al.*, 1998) wherein a complete loading module from the
20 avermectin PKS has been swapped with the erythromycin loading module, while keeping the rest of the DEBS modules intact. As expected, incorporation of butyryl-CoA as well as 2-methylisobutyryl-CoA was seen and in both cases, the end products contained the above mentioned residues. A closely-related experiment has been reported by Kuhstoss *et al.* (1996) in which the loading module from the platenolide PKS was
25 replaced with the loading module from tylactone PKS to yield the expected polyketide product.

It is very clear from the various engineering efforts outlined above that the aim must now be to exploit the potential for genetic manipulation of
30 type I (modular) polyketide synthases (PKS) to produce hybrid synthases

that might catalyse the formation of novel secondary metabolites in a predictable way.

What might be a giant step towards the realisation of this aim, would be to investigate whether these enzymes might be constructed *de novo*, as
5 an essential step in developing a truly combinatorial biosynthesis of complex polyketides.

The 'assembly line' nature of type I polyketide synthases (PKS) that contain sets (called modules) of structurally similar but functionally different enzymatic activities (domains) suggests their potential as a source of "off-
10 the-shelf" enzymatic reagents which can be used to synthesise new and complex polyketide molecules. Outlined below are methodologies for the rapid assembly of DNA units encoding such enzyme domains or modules of enzyme domains.

There are over 40 gene sequences for polyketides that are available
15 from various databases. In addition there are numerous domains known from other synthetic enzymes such as, for example, fatty acid synthase (Joshi and Smith, 1993), peptide synthetases (Elsner *et al.*, 1997) and hybrid polyketide/peptide synthesising enzymes (Paitan *et al.*, 1999; Shen *et al.*, 1999). This amounts to a vast library of domains and modules that
20 cater for a chemical reaction (e.g. stereospecific condensation, dehydration, etc), or in the case of a module, a set of chemical reactions. In order to obtain analogues of a bio-active molecule, research efforts till now have been focused on strategies that involve either chromosomally altering the PKS genes that make the particular molecule (McDaniel *et al.*,
25 1999) or feeding synthetic intermediates to the PKS (Jacobsen *et al.*, 1997). Because of the simplified nature of such experiments, these strategies will remain a fast route towards obtaining a wide variety of drug analogues. However, in the case of compounds like the highly potent anti-cancer discodermolide (TerHaar *et al.*, 1996) the only possible means of obtaining
30 sufficient quantities of the drug is through chemical synthesis. This is because in such cases, the genes responsible for making these bio-active

molecules have not been isolated. The chemical synthesis of large molecules having numerous chiral centres like for example discodermolide, howsoever elegant, is tedious and expensive to scale-up (Marshall and Johns, 1998).

5

Abbreviations

In addition to those listed in *Biochem. J.* (1986) 233, 1-24, the following abbreviations have been used:

10	6-dEB	6-deoxyerythronolide B
	6-MSA	6-methylsalicylic acid
	6-MSAS	6-methylsalicylic acid synthase
	ACP	acyl carrier protein
	AT	β -keto acyl transferase
15	bp	base pair(s) of DNA
	DEBS	6-deoxyerythronolide B synthase
	DH	β -hydroxyacyl-ACP dehydratase (dehydratase)
	ER	enoyl reductase
	FAS	fatty acid synthase
20	kbp	kilobase pair(s)
	KR	β -ketoacyl reductase
	KS	β -ketoacyl synthase
	ORF	open reading frame
	PKS	polyketide synthase
25	RAPS	rapamycin synthase
	TE	thioesterase

The Invention

- In one aspect the invention provides a method of assembling several DNA units in sequence in a DNA construct. This method comprises the
- 5 steps of:
- a) providing each DNA unit with a restriction enzyme recognition sequence at its 5' end and with a recognition sequence for the same restriction enzyme at its 3' end that is combined with a recognition site for a DNA modification enzyme,
 - 10 b) providing a starting DNA construct having an accessible restriction site for the same or a compatible restriction enzyme and cleaving the starting DNA construct with such a restriction enzyme,
 - c) inserting the desired DNA unit and bringing the ligated product into contact with a DNA modification enzyme such that the restriction site at the
 - 15 3' end of the inserted DNA unit is abolished,
 - d) cleaving the ligated product at an accessible unmodified recognition site for the same or a compatible restriction enzyme,
 - e) repeating steps c) and d) to introduce each desired DNA unit to give a DNA construct containing all the desired units in sequence.
- 20 DNA units can be any desired DNA sequence, though usually they encode enzyme domains or modules of two or more enzyme domains. The recognition sequences are usually positioned at the ends of the DNA unit once the DNA unit has been cut with the relevant enzyme, by this it is meant that the recognition sequences are adjacent to the coding sequence,
- 25 or that they flank the said sequence. An accessible restriction site is herein defined as a restriction site which is unmodified, such that it can be cleaved by a restriction enzyme that normally recognises the sequence of the site. The accessible restriction site is preferably a unique site in the DNA unit or ligated product. Where there is more than one accessible site present, it is
- 30 possible to perform a partial digest, as known in the art, to obtain digested products in which only the required site is cleaved in the DNA unit. The

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DNA modification enzyme employed in the method can be a methylase for example the *dam* methylase of *Escherichia coli*. Other methylases such as *dcm* are also envisaged.

A particular method comprises the steps of

- 5 a) providing each DNA unit with an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5' end and with an *Xba*I recognition sequence 5'GATCTAGA3' at its 3' end.
- b) providing a starting DNA construct having an accessible *Xba*I site and cleaving the starting DNA construct with *Xba*I,
- 10 c) inserting the desired DNA unit and using a resulting ligated product to transform a *dam*⁺ strain of *E. coli*,
- d) recovering a resulting plasmid and cleaving the plasmid at an accessible *Xba*I site with *Xba*I,
- e) repeating steps c) and d) to introduce each desired DNA unit to give a
- 15 DNA construct containing all the desired units in sequence.

The recognition sequences for the restriction enzyme and the DNA modification enzyme employed in the method can be created in the DNA units prior to cutting with the restriction enzyme, for example by means of a primer extension reaction. The preferred DNA construct made by the

20 method can be an expression vector capable of facilitating expression of the protein encoded by the desired DNA units.

It is also envisaged that the DNA modification can be removed and the restriction site re-established by replicating the ligated product in a *dam*⁻ strain of *E. coli* by means of suitable vectors as known in the art.

25 The invention also encompasses DNA unit assemblies where any given restriction enzyme recognition site can be modified by addition of a certain combination of nucleotide bases in order for it to be protected.

In a further aspect, the invention provides a method of making an assembly of several DNA units in sequence which method comprises the

30 steps of:

- 9 -

- a) providing a first DNA unit with a recognition sequence for a first restriction enzyme at its 3' end, and cleaving the said first DNA unit with said first restriction enzyme,
- b) providing each other DNA unit with a recognition sequence at its 5' end for a second restriction enzyme which has a compatible ligation sequence with that of the first restriction enzyme, and an upstream recognition sequence for said first restriction enzyme and a downstream recognition sequence for a third restriction enzyme at its 3' end, and cleaving each said other DNA unit with the second and third restriction enzymes,
- 10 c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product such that the ligation of the two units abolishes the recognition site for the first restriction enzyme at the ligation junction, and cleaving the ligated product with said first restriction enzyme,
- d) ligating the product from c) with a desired DNA unit from b) to form a
- 15 ligated product and cleaving the ligated product with said first restriction enzyme
- e) repeating step d) with each other DNA unit in turn so as to assemble the DNA units in sequence.

A particular method comprises the steps of:

- 20 a) providing a first DNA unit with an *Xba*I recognition sequence 5'TCTAGA3' at its 3' end, and cleaving the said first DNA unit with *Xba*I,
- b) providing each other DNA unit with a *Spe*I recognition sequence 5'ACTAGT3' at its 5' end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence
- 25 5'CCCGGG3' at its 3' end, cleaving each said other DNA unit with *Spe*I and *Sma*I, and dephosphorylating the 5' end of the cleaved DNA unit,
- c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product and cleaving the ligated product with *Xba*I,
- d) ligating the product from c) with a desired DNA unit from b) to form a
- 30 ligated product and cleaving the ligated product with *Xba*I

e) repeating step d) with each other DNA unit in turn so as to assemble the DNA units in sequence.

In one embodiment the assembly can occur via stepwise addition of fragments to a vector.

5 In an alternative embodiment the first DNA unit can be attached to the solid phase for use in step c). This permits the solid phase to be split and mixed between steps c), d), and e) to make several different assemblies. Methods of attaching DNA units to the solid phase are well known in the art. Preferred solid phase elements are beads attached to the
10 DNA units via a biotinylated nucleotide, as known in the art.

The recognition sequences in one or more of the DNA units are preferably introduced by means of extension primers, as known in the art, though other methods such as the ligation of the required sequences or *in vitro* mutagenesis can also be employed.

15 The assembly of several DNA units can be inserted into an expression vector and thus used to transform a host capable of expressing the protein encoded by the insert of the vector.

The method is particularly useful where one or more of the DNA units encodes a catalytic or transport protein domain for example a
20 ketoreductase domain from a PKS enzyme or an ACP domain from a hybrid polyketide/peptide synthesising enzyme. Such domains can be derived from enzyme domain DNA sequences from, for example, polyketide synthesising enzymes, peptide synthesising enzymes, hybrid peptide polyketide synthesising enzymes, fatty acid synthesising enzymes
25 or other enzyme domains known in the art.

The DNA units used in the methods of the invention can encode modules comprising one or more catalytic or transport domains. Usually a module contains all of the domains required to complete one condensation step in the synthesis of a target molecule.

30 Alternative aspects of the invention resulting from the methods of the invention include: DNA constructs or vectors incorporating a DNA assembly

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encoding synthetic enzymes, synthetic enzymes encoded by such DNA assemblies, hosts expressing synthetic enzymes, hybrids of transformed hosts expressing synthetic enzymes, and compounds produced by the synthetic enzymes.

5 Where the product produced by the synthetic enzyme exhibits toxicity to a host strain, this can be worked around e.g. by means of choosing a different strain or mutating the original strain to provide mutants which are more tolerant. The diversity of compounds produced by hosts transformed with the synthetic enzymes of the invention can be further increased by
10 using known methods of using different feedstocks in the fermentation to provide different starter units for the desired product. Where yield of desired synthetic enzyme product is low, routine steps e.g. mutation and selection, can be taken to improve this,

 The synthetic enzymes of the invention can also be used in cell-free
15 systems to produce the desired target molecule *in vitro* as known in the art, for example, see Carreras and Khosla (1998).

 In a further aspect, the invention provides a method of synthesising a target molecule comprising the steps of
a) examining the composition and stereochemistry of a target molecule,
20 b) determining which catalytic and transport domains need to be present in a synthetic enzyme in order to catalyse the synthesis of the target molecule,
c) using any one of the methods of the invention to assemble the required DNA units encoding the catalytic and transport domains into a DNA
25 assembly that encodes said synthetic enzyme which is capable of synthesising the target molecule.
d) placing the DNA assembly into a vector to allow expression of the synthetic enzyme in a host capable of synthesising the target molecule after transformation with said vector.

30 Target molecules are generally bio-active molecules, usually having a predominantly carbon based backbone and usually are macromolecules

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comprised of condensed units. The transformed host can be tested for the presence of the target molecule after step d). If yields of the desired compound are low then conventional methods of improving product yield from, for example Streptomyces, can be employed. Transformed hosts
5 which result from the methods of the invention and their use in producing target molecules are also aspects of the invention. Hosts suitable for transformation with the DNA assemblies of the invention are known in the art and include insect or mammalian cells, though more usually suitable are bacterial cells, for example, the improved host strains described by
10 Ziermann and Betlach (1999).

As stated previously, it is also envisaged that the synthetic enzyme can be used in a cell-free system to produce the target molecule *in vitro*.

A further aspect of the invention is a method of making a synthetic
15 enzyme to catalyse the synthesis of a target molecule comprising the steps of

- a) examining the composition and stereochemistry of a target molecule,
- b) determining which catalytic and transport domains need to be present in the synthetic enzyme in order to catalyse synthesis of the target molecule,
- 20 c) using any one of the methods of the invention to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes an enzyme which is capable of synthesising the target molecule.
- d) expressing the DNA assembly in a suitable host to produce the enzyme.

25 In a further aspect the invention provides a library of DNA units encoding catalytic or transport protein domains, wherein each DNA unit has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

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In a particular embodiment of such a library, each DNA unit has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end

Also provided by the invention is a library of DNA units encoding
5 catalytic or transport protein domains, wherein each DNA unit has a recognition sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed by a downstream recognition sequence for a third restriction enzyme at its 3' end, such that the DNA units, once restricted by the first and second
10 restriction enzymes can be ligated together to abolish the restriction sites at the ligation junction. In one embodiment of this aspect of the invention each DNA unit has a *Spe*I recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3'-end

15 Catalytic or transport protein domains can be derived from any enzyme, for example those listed above. Particularly envisaged are libraries in which the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

20 Also provided by the invention are modules comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA
25 modifying enzyme. An envisaged module has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end

Alternatively a module comprising a DNA sequence encoding a functional set of polyketide synthetic domains can have a recognition
30 sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed by a

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Also envisaged are transformed hosts which prior to transformation with a vector containing one or more modules according to the invention, were already lacking a *recA* function.

In a further aspect the invention provides kits containing DNA units,
5 DNA modules, vectors, DNA manipulation hosts, DNA modification hosts, expression hosts, or solid phase elements for use in the methods of the invention. For example, one such kit might contain a first DNA unit which is a vector suitable for transforming a suitable host, a library of modules for insertion into that vector, both the first DNA unit and the library having the
10 necessary recognition sites for use in the methods of the invention, together with host strains suitable for the manipulation and expression of the DNA assemblies of the invention.

A *de novo* "domain-by-domain" reconstruction of a hybrid multienzyme from the erythromycin-producing PKS has been achieved by
15 the inventors by assembling DNA units corresponding to the constituent domains. The assembled gene was expressed in *S. erythraea* and the expected compounds were isolated from the bacterial broth. Application of this methodology, or variations of this methodology for making combinatorial assemblies of complex and aromatic PKSs allows for the
20 rapid generation of novel or altered PKS or other synthetic multienzymes and paves the way for a quick and inexpensive synthesis of potentially bio-active molecules.

One alternative to chemical syntheses is to carry out a 'retrobiosynthetic analysis' of the desired molecule, by pinpointing the exact
25 number and type of synthetic enzyme domains that are required for every chemical step, and then assembling the DNA units that encode these enzymes in order to make a hybrid synthetic enzyme. The aim is therefore, to assemble these domains or even modules in a manner as desired, so that the linked enzymes can carry out a progressive synthesis of a desired
30 target molecule. Until now, it has not been possible to find a methodology to assemble these PKS DNA units using restriction enzymes and DNA

ligase to cut and join the DNA pieces together - one of the limiting factors being the non-availability of appropriate restriction enzyme sites in the DNA sequence of the enzymes which synthesise these polyketide drugs. There exist very few unique restriction enzyme sites and even fewer restriction enzymes that do not cut in the polyketide DNA sequence (i.e. are "non-cutters"). However, the restriction enzyme *Xba*I, because of its TA-rich recognition sequence (5'TCTAGA3'), does not cleave the majority of GC-rich polyketide gene clusters. Thus, flanking both ends of the DNA of the desired DNA unit (domain or module) with a recognition sequence that is cleaved on one end by *Xba*I, and on the other end by a restriction enzyme that is compatible with *Xba*I (e.g. *Spe*I) is possible. A vectorial assembly, where such units are progressively joined, leaves one end of the unit that has been constructed by the ligation of *Xba*I and *Spe*I-cut DNA ends, not recognisable by either of the two enzymes, thus making further addition of units possible at only one of the two ends.

This strategy makes use of selective recognition of the restriction enzyme site by the restriction enzyme *Xba*I, depending upon the sequence adjacent to the restriction enzyme site and upon the strain used (*dam*⁺ or *dam*⁻) during the assembly process. The method has been shown to be successful, and by using this methodology to assemble modules, the complete erythromycin-producing PKS (comprising of six modules coded by three large open reading frames) can be built in under 10 days. Even though this time-period is small compared to what it would take to assemble the *ery* PKS genes using conventional methodologies, using a variation of the above mentioned methodology, complete gene-clusters, like the 33 kbp erythromycin PKS, can be built within a matter of hours.

Also described herein, is an approach wherein the assembly of the units itself can also be carried out *in vitro* without the need for an *in vivo* DNA modification step. Furthermore, employing the *in vitro* assembly methodology described below, one is now able to not only construct predetermined PKS genes, but also a randomly constructed combinatorial

library of shuffled domains from one or more known synthetic enzymes.
This has immediate and important implications for drug-discovery.

The methodology thus outlined requires DNA units to be modified so that they contain the appropriate 5' and 3' ends (X and X^d respectively).

5 These units are then progressively assembled to achieve the desired gene length. The vector containing the assembled or reconstructed gene is then used to transform an expression system to achieve protein expression. This methodology has been shown to work effectively - the hybrid multienzyme DEBS1-TE was reconstructed by assembling *de novo* the ten
10 constituent domains. The assembled gene, when expressed in *S. erythraea* gave the expected six-membered triketide lactones.

However, in the case of larger molecules like discodermolide, one would require a vectorial assembly of some 50 or so PKS units (if domains). A hypothetical PKS that would make a molecule as large as
15 discodermolide would require 12 modules, each possessing the appropriate KS, AT, ACP and a set of reductive domains (e.g. KR, DH or ER). One would find that some of the domains in this group of 50 would be required to carry out the same catalytic function. For example, if all the hydroxy groups resulting from the ketoreductase activity from all 12
20 modules are of the same configuration, in effect 12 KRs that function in an identical fashion are required. Also, all 12 ACPs would, of course have the same catalytic function. It would therefore logically be more convenient, and less time-consuming if, to achieve ketoreduction from every one of the 12 modules, one used only one KR domain instead of 12 different ones in
25 all the modules, or one ACP instead of 12 different ACPs. In fact, one can calculate that for every possible chemical reaction that can be carried out using PKS domains, one requires a set of only 12 domains, that in theory can be used repeatedly (Figure 1).

It is possible that inter-modular recombination events within the
30 reconstituted PKS or other synthetic enzyme gene, may preclude the use of identical PKS or other enzyme domain DNA units in a set of modules. It

might be expected that, for example (Figure 2) the ACP* DNA in module 1 to recombine with the identical ACP* DNA in module 3. This event can take place, for example, when the expression vector that possesses the assembled gene containing numerous identical PKS DNA units is used to transform a streptomyces host for polyketide production.

The inventors have developed a strategy that can circumvent this problem, therefore making it possible to construct large synthetic enzyme gene clusters using identical domains or modules *repeatedly*. This translates into a less expensive route towards synthetic enzyme gene construction (one would not require to have a start-up library of 200 or so to cover all possibilities), as the set of 12 domains, or similar functional arrangements of domains, are true "off-the-shelf" components for the assembly of PKS genes or genes for other hybrid synthetic enzymes.

The inventors provide methods of DNA assembly that pave the way for a cheap and fast synthesis of a host of bio-active molecules, e.g. the anti-cancer drug Discodermolide.

The examples that follow are better described with reference to the following figures:

Figure 1 shows the chemical/stereochemical choices that each PKS domain can make. A total of 12 domains are required for every conceivable polyketide reaction.

Figure 2 shows integration of a plasmid containing more than one identical DNA unit (ACP*). After the plasmid has integrated in the streptomyces host through homologous recombination with TE, internal recombination can occur to yield truncated PKS genes. This is because the host is $recA^+$.

Figures 3A and 3B show a schematic representation of the assembly process. The *de novo* construction of DEBS1-TE. DNA fragments (units) encoding for the constituent domains of the multienzyme DEBS1-TE were inserted sequentially into the expression plasmid pCJR24.

The final plasmid pAR10 was then expressed in *S. erythraea*/JC2 to yield the expected triketide lactone products that are synthesised by the schematically shown re-assembled DEBS1-TE synthase. The amino acid changes made within the linker regions between domains are shown below the actual amino acid sequence. Construction of the expression plasmid pAR10 and structural characterisation of the two triketide lactones shown in the above figure is described in the *methods* section. X – *Xba*I restriction enzyme recognition sequence (5'TCTAGA3'), X^d – *Xba*I and *Dam* methylase recognition sequence (5'GATCTAGA3')

Figure 4 shows the methodology of the assembly of DNA units using *Xba*I/*dam* methylase technology. During the second last stage of assembly, indicated as transform and cut in the figure, transformation of a *Dam*⁻strain with plasmid (as it is a *dam*⁻strain, even X^d would be cleaved by *Xba*I) is effected. Cutting is achieved by *Xba*I and the DNA unit purified on a gel.

Figure 5 shows the procedure for the assembly of DNA units using *Xba*I/*dam* methylase technology.

Figure 6 shows how an *Xba*I site can be made sensitive to methylation. The RE cuts at the sites shown by arrows. The boxed sequence is methylated in a *dam*⁺strain thereby altering the *Xba*I recognition site. The sequence however is not methylated in a *dam*⁻strain, and so can still be cleaved by *Xba*I. The *Xba*I recognition sequence (5'TCTAGA3') can therefore be selectively cleaved by *Xba*I. Assembly of DNA units uses only one restriction enzyme – *Xba*I.

Figure 7 shows the methodology of the *in vitro* assembly of DNA units – I using solid phase beads with the enzymes *Xba*I, *Spe*I and *Sma*I (other *Xba*I – compatible REs may be used).

Figures 8 and 9 show how the methodology of the *in vitro* assembly of DNA units – It would proceed to the point of placing the DNA assembly into an expression vector for transforming and appropriate host. *In vitro* assembly of DNA units (domains) from the first multienzyme of erythromycin – producing
5 PKS.

Figure 10 shows how in one single ligation, 16 ongoing assemblies are generated. This cascade can obtain exponential proportions. The gene library can be increased by increasing the diversity of the incoming unit.

10 Figure 11 shows the integration of an expression plasmid into a streptomyces host, using a mutated internal fragment of the *recA* gene as the region for homologous recombination. The resulting PKS gene can now contain more than one identical DNA units as the strain has been made *recA* minus.

15 Figure 12 shows the assembled PKS *recADEBS1-TE*. The second module is composed of domains that normally belong to the first module.

Figure 13 shows the amino acid sequence alignment of the *recA* protein of *S. lividans* (S.l.) and *S. ambofaciens* (S.a). Percent similarity: 96.496, percent
20 identity: 95.418. Match display thresholds for the alignment(s):

I = identity

: = 2

. = 1

25 Figures 14A and 14B show a DNA sequence alignment of the *recA* gene *S. lividans* (S.l.) and *S. ambofaciens* (S.a). Start of the gene is from 'ATG' and stop is 'TGA'. Percent similarity: 94.713, percent identity: 94.713.

30 Figure 15 shows how an *XbaI/SpeI* system might be used instead of an *XbaI/dam* methylase system to assemble DNA units, a strategy involving compatible restriction enzymes flanking either end of a DNA unit. An example

of compatible REs would be *Xba*I and *Spe*I. The recognition sequence of *Xba*I is – 5'TCTAGA3' and that for *Spe*I is 5'ACTAGT3'. After *Xba*I and *Spe*I have cleaved the DNA at their respective sites, the DNA unit can be ligated together as the overhanging is complementary. The junction where any two
5 units are joined is now recognised by either *Xba*I or *Spe*I.

Figure 16 is a schematic representation of the compatibility of *Xba*I- and *Spe*I-digested DNA overhangs. It shows the compatibility of the sticky ends produced by *Xba*I and *Spe*I and how re-ligation abolishes both sites.

10 Figure 17 shows a schematic representation of the erythromycin-producing polyketide synthase; primary organisation of the genes and their corresponding protein domains. The multienzymes deoxyerythronolide B synthase 1 (DEBS1), DEBS2 and DEBS3 each have two modules, each of which processes one cycle of polyketide chain extension. Each of the six
15 modules is constituted by covalently-linked enzymatic domains. Exploitation of such an enzymatic hierarchy as "of-the-shelf" reagents can lead to synthesis of important chemical compounds.

Figure 18 shows the structure of the anticancer drug discodermolide (top) and
20 the 'retrobiosynthetic approach' towards synthesising a target molecule (a discodermolide). Such an approach would involve opening up the structure (a.), identifying the number and type of polyketide carbon units that would make the discodermolide carbon skeleton (b.), and choosing the PKS DNA units (modules/domains) responsible for the uptake and subsequent
25 processing of the carbon units (c.).

Figure 19 shows the anti-tumour compound octalactin and the strategy behind the retrobiosynthetic approach towards synthesising bio-active molecules. The strategy comprises the steps of:

Identify polyketide units – e.g. whether acetate, propionate, etc,

Break-up and identify – break up the carbon skeleton and identify how many such carbon units are present. Eight units would mean one requires eight
5 modules to make a PKS.

Choose – choose the modules or domains that would be required, form an existing library of such PKS modules and domains.

10 Assemble – assemble the DNA units (modules/domains) using the invention.

Express – express the assembled gene in a host and check for compound production.

15 Figure 20 shows a schematic representation of they hypothetical polyketide synthase for synthesising octalactin B, assembled from enzyme units that belong to various PKSs in the public domain.

Figure 21 shows a schematic representation of the hypothetical
20 decarestrictine polyketide synthase for synthesising the anti-cholesterol compound decarestrictine J, assembled from enzyme units that belong to various PKSs in the public domain.

APT 34 AMDT

Examples

Example 1: Vectorial assembly of DNA units

DNA units that are to be assembled contain the *Xba*I recognition sequence at either end of the unit. At one of the ends, two nucleotides (GA) are arranged at the 5' end of the *Xba*I recognition sequence (thus making it 5'GATCTAGA3'). This is achieved by first incorporating the *Xba*I recognition sequences in the oligonucleotide primers and then amplifying the desired DNA unit by PCR. The PCR products are then ligated to a pUC-18 vector, used to transform a *dam*⁺ strain of *E. coli*, and the clones isolated and sequenced for possible errors in the PCR products. A *dam*⁺ strain of *E. coli* - like DH10BTM - methylate the nucleotide A in the sequence GATCTAGA, as 5'GATC3' is a sequence that is recognised by the product of the Dam methylase gene (Fujimoto *et al.*, 1965; Geier *et al.*, 1979). This makes only one end of the DNA unit cleavable by *Xba*I. The vector is then used to transform a *dam*⁻ strain of *E. coli* (e.g. ET12567 - MacNeil *et al.* (1992)) and the plasmid DNA isolated. This DNA is now cleavable at *both* ends of the DNA unit by *Xba*I. When a library of units has been constructed using this strategy, and both ends of these units have been cleaved by *Xba*I, they are progressively inserted into a vector that has a unique *Xba*I site and the ligated products are used always to transform a *dam*⁺ strain of *E. coli*, thereby making sure that one end of the DNA unit is always protected from cleavage by *Xba*I through methylation. When the assembly of such units is completed, the final plasmid is integrated into a streptomyces strain for the production of the desired polyketide.

Using this methodology, the polyketide synthase DEBS1-TE, a multienzyme that has the first of the three bimodular erythromycin DEBS enzymes (DEBS1), fused with the erythromycin thioesterase (Cortés *et al.*, 1995) was constructed in a *de novo* fashion. The ten inherent PKS domains in DEBS1-TE, namely, loading module (itself composed of an AT and an ACP), KS1 (ketosynthase of module 1), AT1, KR1, ACP1, KS2

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(ketosynthase of module 2), AT2, KR2, ACP2 and TE function in conjunction to catalyse the synthesis of (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-hexanoic acid δ -lactone (2), figure 3.

The DNA for all ten domains was amplified by PCR to incorporate the two aforementioned recognition sequences for *Xba*I (5'TCTAGA3' and 5'GATCTAGA3') at the 5' and 3' ends of the DNA unit respectively. The PCR products were cloned in pUC18 vector, sequenced, and then used to transform the *dam*⁻ *E. coli* ET12567 strain. To initiate the assembly process, the DNA unit for TE was inserted into *S. erythraea* expression vector pCJR24 (Rowe *et al.*, 1998) which has a unique *Xba*I site. This vector also contains a thiostrepton-resistance gene as a marker for identifying successful integrands. The ligated products were used to transform the *dam*⁺ *E. coli* DH10BTM strain and the plasmid DNA isolated. This plasmid (pAR1) can only be singly cleaved with *Xba*I, despite possessing two *Xba*I recognition sequences, as one of the sites (situated at the 3' end of the TE unit) has been methylated by the *E. coli* Dam methylase. The next DNA unit (ACP2 from module 2 of DEBS1) was then ligated to the *Xba*I-cut pAR1, the ligation mixture used to transform DH10B cells and the plasmid DNA isolated. Likewise, the other eight DNA units were successively added to pAR1 to finally yield the expression plasmid pAR10 containing the reconstituted DEBS1-TE gene (Figure 3). The junctions where these domains were joined were chosen in the linker regions that lie between these domains, so as to cause minimum disturbance of the structural features of these domains, that might in turn affect the proficiency of the domains themselves (Figure 3). Plasmid pAR10 was then used to transform *S. erythraea*/JC2 - a mutant strain of the wild-type *S. erythraea* NRRL2338 that lacks the DEBS genes except for the TE DNA fragment (Rowe *et al.*, 1998). Thiostrepton-resistant colonies were selected upon integration of the vector into the *S. erythraea* chromosome. Single transformants were grown on selective media, as described in the *methods* section. The fermentation broth was extracted with ethyl acetate

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and a sample of the organic extract was analysed by gas chromatography-mass spectroscopy (GC-MS). Two peaks were observed, corresponding to molecular masses 158 and 172, indicating the presence of the expected acetate- and propionate- derived polyketides (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-pentanoic acid d-lactone (1) and (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-hexanoic acid d-lactone (2). Both compounds were isolated and fully characterised by high-pressure liquid chromatography (HPLC), ¹H 1D and 2D NMR, ¹³C NMR, FT-ICR spectrometry, and by comparison with a synthetic standard of (2) (Brown *et al.*, 1995). One litre of fermentation broth produces 24 mg of (1) and 56 mg of (2) - yields that are comparable to those reported elsewhere (Lau *et al.*, 1999). It can therefore be asserted that the ten newly constructed inter-domain junctions have not in any way dimmed the catalytic proficiency of the DEBS1-TE synthase.

In the absence of any crystal-structure data on PKS domains, all genetic engineering efforts known in the art have been based on trial-and-error methods of experimenting with where to join two such domains. As a result, the yield of the synthesised polyketide products have varied depending upon the position in the polypeptide chain at which the domains or modules have been linked (McDaniel *et al.*, 1999; Ruan *et al.*, 1997). The successful functioning of the reconstructed polyketide synthase described above has supplied new information about the inter-domain junction sites. Using this information, and the described methodology for the rapid assembly of these enzyme units, it is now possible to carry out a 'retrobiosynthetic analysis' of target molecules and then to use polyketide and other biosynthetic enzyme domains as truly 'off-the-shelf' reagents to achieve a stereospecific synthesis. There is also the possibility of using this methodology for randomly combining DNA units that encode catalytic e.g. DH or transport e.g. ACP protein domains to generate combinatorial libraries of hybrid synthases. By using a suitable assay system to test for biological activity of the compounds that are generated by such means, it is

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possible to go back and isolate the hybrid synthetic gene responsible for the production of these compounds.

From 6-methylsalicylic acid to maitotoxin, nature displays a staggering diversity in compounds that are synthesised by means of 'combinatorial gene-shuffling'. This methodology, or variations of this methodology can be used as effective tools towards harnessing the combinatorial potential of discrete enzymatic units or their sets that are the feature of multi-functional PKS and other systems.

A similar system to the *XbaI/dam* system described above, uses the restriction enzyme *FokI* which has the recognition site:

5'GGATG(N)₉↓3'

3'CCTAC(N)₁₃↑5'

with the *dcm* methylase of *E.coli*. Adding CCA or CCT to the 5' end of the *FokI* recognition site would make the site *dcm* sensitive. Furthermore, if the sequence TCTAGA were inserted into the redundant section of the *FokI* restriction site, then the enzyme could be used to generate 'XbaI-cut ends'.

Methods

E. coli dam⁺ DH10BTM strain was purchased from Gibco BRL, USA.. *Pfu* DNA polymerase was purchased from Boehringer, Germany.

Construction of the final expression plasmid pAR10 was carried out in several steps, as follows. The ten PKS DNA units were amplified by PCR using *pfu* DNA polymerase. The respective regions of *eryAI* gene, as well as the oligonucleotides used for each PCR are outlined:

LM - segment of *eryAI* gene (Bevitt *et al.*, 1992) extending from nucleotide (N) 588 to N 2389;

5'GGCATATGGCGGACCTGTCAAAGCTCTCCGACAGT3' and

5'GGTCTAGATCCCAGCCGCGGTCTGGTCTGGCAGTCCCG3',

KS1 - segment of *eryAI* gene extending from N 2384 to N 3769;

5'GGTCTAGACTCGCTGTTCCACCCCGACCCACGCGCTCGGGCACC

GCGCACCA3' and

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5'GGTCTAGATCGCGCAGCGCGGCGGACTCGTCGACGGGGGCGAAG
GCGG3',

AT1 - segment of *eryA1* gene extending from N 3764 to N 4813;

5'GGTCTAGACGGTCTCGCGACGGGAAACGCCGACGGTGCCGCCGTT
5 GGAA3'

and

5'GGTCTAGATCCACCGCGACACCGGCGGGCGAACGCGCGGGAGAGC
GCTTCGC3',

KR1 - segment of *eryA1* gene extending from N 4808 to N 6316;

10 5'GGTCTAGAGTCGGTGCACCTGGGCACCGGAGCACGCCGGGTGCCC
TT3'

and

5'GGTCTAGATCGTCGAAGAGCCTGGTCGGGCGCTGCGCGGTGTA3',

ACP1 - segment of *eryA1* gene extending from N 6311 to N 6679;

15 5'GGTCTAGACGACGCGCGGCGGGCTGCGCCGCAGGCGCCGGGCCGA
ACCGCGGG3'

and

5'GGTCTAGATCGGCCGTGG-TCGCCGGTGCCGCCTGCTCGGCT3',

KS2 - segment of *eryA1* gene extending from N 6674 to N 8200;

20 5'GGTCTAGACGAGCCGATCGCGATCGTCGGCATGGCGTGCG-
CGGCTGC3'

and

5'GGTCTAGATCGTGACGGCCTCGGCGGTGTCGGCGGCGAGC-
ACCGCGGCCCGCTCCTC3',

25 AT2 - segment of *eryA1* gene extending from N 8195 to N 9340;

5'GGTCTAGAGGCGGTGGCCGACGGCGCGGTGGTT3'

and

5'GGTCTAGATCGTCACGAGGGGTGGTGCGGTCCGGCAGCAGCCAGA
A3',

30 KR2 - segment of *eryA1* gene extending from N 9335 to N 10639;

5'GGTCTAGACGGCTGGTTCTACC-GGGTCGACTGGACCGAG3'

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and

5'GGTCTAGATCCGGCCGGGGCCGGGCGGCGG-TGTAGGACT3',

ACP2 - segment of *eryA* gene extending from N 10634 to N 10966;

5'GGTCTAGACCGCATCGTCACGACCGCGCCGAGCGA3'

5 and

5'GGTCTAGATCG-GCGTCGAGGAAA3',

TE - segment of *eryAIII* gene (Donadio *et al.* 1991) extending from N 8753 to N 9602; 5'GGTCTAGACAGCGGGACTCCCGCCCGGGAAGCG3'

and

10 5'GGGCTAGCTCTAGATCATGAATTCCCTCCGCCCAGCCAGGCGTC3'.

All PCR products were 5' phosphorylated and ligated to *Sma*I-cut, dephosphorylated pUC18 vector and used to transform *E. coli* DH10B electrocompetent cells. The desired plasmids - containing the amplified DNA fragments were isolated and sequenced using standard pUC forward and reverse primers. No mistakes in the amplified products were detected.

15 All ten plasmids were then used to transform the *E. coli* ET12567 *dam*⁻ strain. Isolated DNA was digested with *Xba*I restriction enzyme and desired fragments isolated and purified. The TE unit was then ligated to *Xba*I-cut pCJR24 vector and the ligation products used to transform *E. coli* DH10B

20 electrocompetent cells. Plasmid pAR1 was isolated, digested with *Xba*I, and ligated to the ACP2 fragment, and ligation products treated as mentioned above. The other DNA fragments, namely, KR2, AT2, KS2, ACP1, KR1, AT1 and KS1 were sequentially added to finally yield plasmid pAR10. This plasmid was then digested with *Nde*I and *Xba*I restriction

25 enzymes and ligated with the LM fragment previously digested with the same two enzymes. The ligated products were used to transform *E. coli* DH10B electrocompetent cells and the final expression plasmid pAR10 isolated. Plasmid pAR10 was then used to transform *S. erythraea*/JC2 strain and colonies carrying the expression plasmid were selected through

30 resistance to thiostrepton upon integration of the plasmid into the *S. erythraea* chromosome. Single transformants were picked and grown on

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tap-water medium plates supplemented with thiostrepton, following which single transformants were grown in 5X200ml of SM3 liquid media supplemented with 5 ug/ml of thiostrepton for seven days (Rowe *et al.*, 1998). Cells were removed by centrifugation, the supernatant was

5 saturated with NaCl and extracted three times with equal volumes of ethyl acetate at pH 4.0. The solvent was evaporated to yield 1.12 g of crude product. A sample of this crude product was analysed by GC-MS. Two peaks were observed, corresponding to molecular masses 158 and 172, indicating the presence of the expected acetate- and propionate- derived

10 polyketides (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-pentanoic acid δ -lactone (1) and (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-hexanoic acid δ -lactone (2). Compounds (1) and (2) were found to be structurally identical to those reported previously (Cortés *et al.*, 1995).

15 *Characterisation of (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-pentanoic acid δ -lactone (1)*

^1H NMR (CDCl_3 , 500 MHz) δ 4.45-4.35 (1H, dq, J = 6.56 and 1.62 Hz, $\text{C}_5\text{-H}$), 3.8 (1H, dd, J = 10.15 and 4.17 Hz $\text{C}_3\text{-H}$), 2.45-2.70 (1H, br, O-H), 2.42 (1H, dq, J = 10.0 and 6.97 Hz $\text{C}_2\text{-H}$), 2.05 (1H, m, $\text{C}_4\text{-H}$), 1.37 (3H, d, J = 7.17 Hz, $\text{C}_2\text{-CH}_3$), 1.32 (3H, d, J = 6.74 Hz, $\text{C}_5\text{-CH}_3$), 0.95 (3H, d, J = 7.20

20 Hz, $\text{C}_4\text{-CH}_3$) ppm. ^{13}C NMR (CDCl_3 , 250 MHz) δ 174.20, 76.15, 73.62, 39.42, 38.14, 18.11, 14.24, 4.48.

Characterisation of (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-hexanoic acid δ -lactone (2)

^1H NMR (CDCl_3 , 500 MHz) δ 4.13 (1H, ddd, J = 8.12, 5.93 and 2.19 Hz, $\text{C}_5\text{-H}$), 3.82 (1H, m, $\text{C}_3\text{-H}$), 2.42-2.50 (1H, dq, J = 10.17 and 7.08 Hz, $\text{C}_2\text{-H}$),

25 2.12-2.19 (1H, m, $\text{C}_4\text{-H}$), 1.77-1.86 (1H, m, one of $\text{C}_6\text{-H}_2$), 1.52-1.61 (1H, m, one of $\text{C}_6\text{-H}_2$), 1.4 (3H, d, J = 7.09 Hz, $\text{C}_2\text{-CH}_3$), 1.0 (3H, t, J = 7.42 Hz, $\text{C}_6\text{-CH}_3$), 0.97 (3H, d, J = 6.96 Hz, $\text{C}_4\text{-CH}_3$) ppm. ^{13}C NMR (CDCl_3 , 250 MHz) δ 173.56, 81.34, 73.96, 40.08, 36.76, 25.27, 14.27, 9.88, 4.37.

30

Example 2: *in vitro* assembly of DNA units

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Figure 7 outlines the strategy for the *in vitro* assembly of PKS DNA units. The inventors have constructed the multienzyme DEBS1-TE. The *in vivo* construction of the gene for DEBS1-TE, it should be noted, took 12 days to complete. The *in vitro* assembly on the other hand was completed
5 in 2 days.

All ten domains, namely, LM, KS1, KR1, AT1, ACP1, KS2, AT2, KR2, ACP2 and TE were amplified by means of PCR. The forward primer in all cases, except the LM contained the *SpeI* recognition sequence 5'ACTAGT3' while the reverse primer was engineered in such a way that it
10 contained the *XbaI* recognition sequence 5' TCTAGA3' and *SmaI* recognition sequence 5'CCCGGG3' downstream of the *XbaI* site (Figure 7). The amplification of the LM was carried out using a biotinylated forward primer and a reverse primer that contained the *XbaI* recognition sequence (5'TCTAGA3'). All the PCR products were cloned in pUC-18 vector and the
15 resulting plasmids sequenced to detect possible errors introduced by PCR. All plasmids, except the one containing the LM unit were then digested with *SpeI* and *SmaI*, dephosphorylated in order to remove the 5' phosphate group and the appropriate fragments isolated and eluted. The LM unit was cleaved with *XbaI* and attached to a bead that was coated with streptavidin
20 (following the manufacturer's instructions) as shown in figure 7.

The assembly process was initiated by adding DNA ligase to the tube containing a large excess of the first unit (KS1) and LM-bead. The reason for having a large excess of the KS1 unit compared to the LM-bead unit is to favour the LM-bead ligating to the incoming unit, as opposed to
25 the self-ligation of the LM-bead (see figure 7). The ligation of the two DNA fragments is unidirectional as only the *SpeI*-cut end of KS1 complements the *XbaI*-cut end of the LM-bead. After the ligation was complete, the desired product of the ligation reaction, namely 'bead-LM-KS1' was separated from the reaction mixture and washed. This product was then
30 cleaved with *XbaI*, in order to activate the 3' end of KS1. The beads were washed again to remove the small *XbaI*-*SmaI* DNA fragment that was

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released from the 3' end of KS1 as a result of RE cleavage. The 'activated' bead-LM-KS1 unit was then ligated with *SpeI*, *SmaI*-cut and 5' dephosphorylated AT1. The *SpeI*-cut 5' end of AT1 complemented the *XbaI*-cut 3' end of KS1 to give bead-LM-KS1-AT1 as shown in figure 8.

- 5 This product was separated from the reaction mixture and washed as before. The 3' end of AT1 in this product was then 'activated' through cleavage by *XbaI*, and the assembly process continued.

- Finally, *SpeI*, *SmaI*-cut and 5' dephosphorylated TE unit was ligated with the DNA fragment that was now bead-LM-KS1-AT1-KR1-ACP1-KS2-
10 AT2-KR2-ACP2 as shown in figure 9. The 3' end of the latter fragment was 'activated' by digesting it with *XbaI*. The assembled DEBS1-TE gene was then inserted in the expression plasmid pCJR24 and the resulting plasmid used to transform a streptomyces strain. The expected triketide lactone products were isolated and structurally characterised.

- 15 Use of the *in vitro* technology described above drastically reduces the time it takes to assemble predetermined or randomly shuffled genes. Also, the possibility of continuing with the assembly process while having numerous different assembly arrays attached to the beads, and splitting and mixing the beads between each unit/module addition from a library of
20 units/modules, results finally in the generation of a cascade of different assemblies (Figure 10). These assembled genes can then be cloned simultaneously and expressed in a suitable host. An assay system can then be used to identify those assembled genes that yield bio-active compounds.

25

Example 3: Retrobiosynthetic synthesis of a target molecule

- A strategy employing the invention in order to construct the highly potent anti-breast cancer drug discodermolide, the anticholesterol compound decastrictine, and the antitumour compound octalacin using
30 polyketide synthase domains/modules is outlined below.

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Discodermolide

The drug discodermolide (Figure 18), isolated from the marine sponge 'Discodermia dissoluta', has been identified as a highly potent anti-cancer compound and 80 times more effective than the well known anticancer
5 drug Taxol (TerHarr *et al.*, 1996). It has the same mechanism of action as Taxol, even though it is structurally different from the latter.

One can infer from its structure (Figure 18) that discodermolide is a polyketide and can therefore be constructed from a system that has the basic enzymatic building blocks (domains and modules) that make other
10 polyketides like erythromycin and rapamycin. Having predicted that approximately 45 domains housed in 12 modules would be required in order to carry out the chemistry that accounts for the functionalities on the carbon skeleton of discodermolide, one can now begin to construct such a system. All one has to do is to identify the type and nature of the
15 domains/modules that one requires to generate the observed functionalities, and then assemble these units in the desired order (Figure 18). The resulting DNA assembly can then be put into a bacterial strain that makes a functional polyketide synthase.

Until now, it would have been exceedingly difficult, if not impossible
20 to assemble 45 or so pieces of DNA in the wanted order, for several reasons. Firstly, one would have to look for two different restriction enzymes every time one needed to assemble two DNA segments. This is because if one uses just one restriction enzyme at either end of the

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domain, the already-assembled piece/pieces of DNA would be cleaved from the assembly every time one decided to insert a new domain. Secondly, in GC-rich DNA like the polyketide synthase producing *Streptomyces* strain, unique restriction enzyme sites are few and far
5 between. To a molecular biologist, the task of assembling 40 pieces of DNA with the limitations mentioned above, would seem an insurmountable one. One would rather attempt to isolate the genes that make the drug at the first place than consider carrying out "step-by-step" reconstruction of the gene itself. In the case of discodermolide, even the last possibility is in
10 the realms of fantasy. The organism within the marine sponge that makes the drug has not been identified. The only way discodermolide can be made available is through chemical synthesis - there have been a few chemical routes reported in literature recently (Marshall and Johns, 1998 and references therein). However, as is the case with most other complex
15 molecules, large scale production of discodermolide, using the chemical route would turn out to be outrageously expensive. Chemists have been using the retrosynthetic analysis approach towards total synthesis of important bioactive molecules. This approach breaks the target compound into many smaller pieces - easily synthesised - which are then re-
20 assembled.

The type of polyketide or other synthetic enzyme domains required in order to construct the target molecule from the starting units are identified using a "retrobiosynthetic analysis" approach for discodermolide,

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by matching which molecules need to be condensed to form the macromolecule with the enzyme domains that carry out the required catalysis to build the macromolecule.

Having identified the enzyme units that are required, the unit-DNA segments are amplified using the polymerase-chain-reaction (PCR) - from the library of existing polyketide synthase unit-DNA, and the appropriate recognition sequences are attached to each unit-DNA fragment. All of the unit fragments are then replicated in a *dam*⁻ strain whereby both the unmodified and modified sequences (5'TCTAGA3' and 5'GATCTAGA3' respectively) are cleaved by the restriction enzyme *Xba*I.

Having constructed this library of appropriate PKS or other synthetic enzyme units, the corresponding DNA units are then assembled. The assembled DNA piece is then placed in a vector, so that it can be inserted in a bacterial strain to yield the desired synthetic protein. Suitable vectors have an antibiotic resistance marker (for selection of this vector on an antibiotic-rich media) and an "origin-of -replication" (ori). Ori is essential for the independent growth of the vector in any strain. Particularly suitable vectors for the expression of the synthetic enzymes of the invention are the actinomycete vectors described by Rowe *et al.* (1998).

The strain is then grown in a media that is supplemented with the antibiotic, the resistance gene for which is present in the vector.

Figures 4 and 5 show how the assembly proceeds. The first domain is inserted into a vector that is cut by cleavage with *Xba*I. After the ligation

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of the domain has taken place with the vector, the DNA is put in a bacterial strain that is *dam*⁺ and grown. Finally, bacterial colonies that have the desired vector-domain DNA are identified and DNA isolated from them. The whole procedure is cheap and fast. Only one restriction enzyme (*Xba*I) is made use of, routine cloning technology is employed, the desired DNA
5 fragment is obtained, which can then be expressed in a *Streptomyces* strain to yield the polyketide synthase.

The *in vivo* "domain-by domain" construction of the discodermolide producing polyketide synthase would take approximately 55 days via this
10 method. In comparison, assembly of modules would take less time, as one would need to assemble fewer pieces. Most importantly, once the synthase is shown to be functionally active, a large fermentation of the bacterial strain can be carried out, and the drug isolated in however much quantity one requires - unlike the chemical route where the starting materials have
15 to be freshly synthesised every time one requires the target compound. Employing such a strategy would lead to a quick and inexpensive synthesis of important bioactive molecules like discodermolide.

Retrobiosynthetic analysis

The whole approach (retrobiosynthetic analysis followed by
20 identification of PKS units, followed by assembly of PKS units) is made clearer in the following two examples.

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Octalactin

A new addition to the rare class of eight-membered lactone natural products is the family of Octalactin. Octalactin A and B (Figure 20) are natural products isolated from the marine gorgonian octocoral '*Pacifigorgia* sp.' (Tapiolas et. al., 1991). Octalactin A shows very strong cytotoxicity toward B-16-F-10 murine melanoma and HCT-116 human colon tumour cell lines and is a promising drug candidate, while octalactine B displayed no such activity (Tapiolas et. al., 1991). Total syntheses of both octalactin A and B have been reported in literature. One such synthesis (Buszek, et. al., 1994) typically involves more than 12 chemical steps in leading to the target molecules. Clearly, large-scale production of octalactins using chemical synthesis is industrially not viable. On the other hand, the genes that code for the enzymes that make octalactins have not be identified or isolated. This means that at present, modified octalactins can only be made using chemical synthesis. A gene is constructed from the available PKS spare parts - that would code for the enzymes that would make octalactin B. Octalactin B can then be converted into the cytotoxic octalactin A by one-step stereospecific epoxidation. Also, once the gene for octalactin B is constructed and shown to make the octalactin PKS, genetic engineering on this gene would yield modified octalactin PKSs that in turn would synthesise octalactin analogues.

Clearly, a polyketide, the carbon skeleton of octalactin B (Figure 19) can be seen to be assembled by acetate and propionate units. The uptake

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and assembly of these units in the prescribed sequence, as well as the functionalities that decorate the carbon chain of octalactin can be assigned to various PKS modules (see figure 19). Once a decision has been made regarding the type and nature of PKS modules, they can be strung together to make a gene using the invention. This gene can then be expressed in a suitable host in order to look for octalactin B production. The retrobiosynthetic approach towards octalactin is shown in detail in figure 19. A choice of what modules to select from the PKS module library is followed by amplification of the modular DNA fragments using the oligonucleotides such that the 5' and the 3' ends of every DNA fragment have the restriction enzyme recognition sites stated under the description of the invention. The choice of modules that, when assembled, would make the 'octalactin gene' is displayed as a schematic representation in figure 20.

15 Decarestrictine J

The molecule decarestrictine J can be synthesised using the retrebiosynthetic approach. Decarestrictine J is a ten-membered lactone that comes from the family of decarestrictines, shown to display strong anti-cholesterol activity (Grabley et. al., 1992). The total synthesis of Decarestrictine J has been reported and involves numerous chemical steps (Yamada et. al., 1995). The target molecule (figure 21) can be conceived to be formed by assembly of five acetate polyketide units. Using the retrobiosynthetic approach, one can identify the PKS domains/modules that

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would be required for the carbon skeleton of decarestrictine J. A hypothetical decarestrictine PKS is shown in figure 21. The loading module, as well as the four internal modules along with the TE domains can be conveniently assembled using the invention. The assembled
5 'decarestrictine gene' can then be expressed in a suitable host in order to check for the production of decarestrictine J.

In summary, the retrobiosynthetic approach involves the following steps;

- a). Identification of the *number* and *nature* of carbon units that make up the
10 target molecule
- b). Identification of the modules/domains from libraries of polyketide/peptide synthetase/fatty acid/etc. encoding units that are responsible for the uptake of the said carbon units and the nature and degree of functionalisation of the carbon chain
- 15 c). Assembly of the said modules/domains using the methods of the invention
- d). Expression of the assembled gene in a suitable expression host.

20 Example 4: Transforming strains with DNA encoding similar synthetic enzyme domains

A method for transforming expression strains with DNA encoding similar synthetic enzyme domains has been devised. Instead of using the TE PKS DNA fragment as a region of integration from the assembled gene into a
25 streptomyces host (*S. erythraea*/JC2, Rowe *et al.*, 1998), a mutated *recA* gene fragment from streptomyces is used. The assembly process is carried

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out in a *recA*⁻ *E. coli* strain (e.g. DH10B) as previously described. As this strain is *recA*⁻, one can assemble any number of identical DNA units. The vector, into which the assembled gene is being constructed, contains a portion of a streptomyces *recA* gene. This *recA* fragment carries a
5 mutation. After the synthetic enzyme gene has been assembled, the vector is used to transform a streptomyces host (e.g. *S. lividans* or *S. erythraea*). The fragment of *recA* gene carrying a mutation recombines with the *recA* gene of the streptomyces host, abolishing the functional *recA* gene and making the strain recombination minus (Figure 11). This means that an
10 event, such as the one described in figure 2 is now not possible. The strain is then grown to look for the encoded enzyme product. This strategy is tested by assembling a functional PKS gene having more than one type of identical DNA units (Figure 12).

Construction of the PKS multienzyme recDEBS1-TE

15 *RecA* protein has been characterised as a multifunctional enzyme that is essential for homologous recombination, DNA repair, SOS response and DNA rearrangements (Miller and Kokjohn, 1990). Most of the routinely used strains of *E. coli* are *recA*⁻. The gene for *recA* has been identified from many streptomyces strains. The first streptomyces *recA* gene to be
20 characterised and isolated was from *S. lividans* (Nußbaumer and Wohlleben, 1994) *RecA* mutants have since been generated in *S. ambofaciens* (Aigle *et al.*, 1997). The streptomyces *recA* protein has approximately 372 amino acid residues (Figure 13). DNA sequence analysis suggests a coding region of 1122 bp, and is found to be highly
25 conserved within streptomyces (Figure 14). In fact the *recA* mutants of *S. ambofaciens* were generated by integrating a mutated portion of the *S. lividans recA* gene into the *S. ambofaciens* host. It was found that a *recA* mutant lacking 30 aa from the C-terminus of the protein inhibited recombination events in *S. ambofaciens* (Aigle *et al.*, 1997).

30 A *recA* mutant of the streptomyces host that is used for expression of the assembled gene was generated.

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The oligonucleotides:

5'- GGTCTAGAATTTCGGCAAGGGCGCCGGTCATGCGCAT-3' and

5'- GGTCTAGATCTGCGGCGTTCGGCCGGGGCGGCGGAGGCG-3'

were used as the forward and reverse primers respectively and the 1000
5 bp internal region of *S. lividans recA* gene (Nußbaumer and Wohlleben,
1994) was amplified using *pfu* polymerase. An additional nucleotide (C)
was incorporated into the forward primer to generate a frame shift in the
amplified *recA* gene fragment. The PCR product was cloned in pUC-18
vector and sequenced to detect for possible errors during PCR. The 1.0
10 kbp *recA* fragment, flanked at both ends by an *Xba*I site was then inserted
in the expression vector pCJR24 that has a unique *Xba*I site. The ligation
mixture was used to transform *E. coli* DH10B cells and the desired plasmid
DNA isolated. The resulting plasmid (pA*RecA*24) contains a non-
methylated *Xba*I site at the 5' end of the *recA* gene fragment. The ten PKS
15 DNA units, namely, TE, two each of ACP1, KR1, AT1 & KS1, and LM were
inserted into the plasmid pA*RecA*24 to finally yield the expression plasmid
p*RecA*D1TE. This plasmid was used to transform wild-type *S. lividans*
protoplasts, and thiostrepton resistant colonies were grown in defined liquid
media as described above. The compound (Figure 12) was isolated from
20 the bacterial broth and chemically characterised.

Thus, it has been shown that a gene carrying interspaced DNA units
that are identical in structure as well as function does not lead to internal
recombination events, as the native *recA* gene of the streptomyces host
has been disrupted. Furthermore, it has been shown that it is possible to
25 use identical domains to reach the objective of generating hybrid synthetic
enzyme systems. This strategy will greatly reduce the number of domains
that otherwise have to be employed for the purposes of *de novo* PKS gene
assembly that yields the desired chemical compounds. The inventors have
established a set of 12 domains that are capable of functioning robustly
30 and are independent of flexibility and spacial constraints - problems that
beset the choice of domains and modules previously.

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References

- Aigle, B., Holl, A-C., Angulo, J.F., Leblond, P. and Decaris, B. (1997) Characterization of two *Streptomyces ambofaciens* *recA* mutants: identification of the *recA* protein by immunoblotting. *FEMS Microbiol. lett.*, 149, 181-187.
- Bevitt, D.J., Cortés, J., Haydock, S.F. and Leadlay, P.F. (1992) 6-Deoxyerythronolide B synthase 2 from *Saccharopolyspora erythraea*. Cloning of the structural gene, sequence analysis and inferred domain structure of the multifunctional enzyme. *Eur. J. Biochem.*, 204, 38-49.
- Brown, M.J.B., Cortés, J., Cutter, A.L., Leadlay, P.F. and Staunton, J. (1995) A mutant generated by expression of an engineered DEBS1 protein from the erythromycin-producing polyketide synthase (PKS) in *Streptomyces coelicolor* produces the triketide as a lactone, but the major product is the nor-analogue derived from acetate as starter acid. *J. Chem. Soc., Chem. Commun.*, 1517-1518.
- Buszek, KR., Sato, N. and Jeong, Y.M. (1994) Total synthesis of octalactin-A and octalactin-B. *J. Amer. Chem. Soc.* 116, 5511-5512.
- Carreras C. and Khosla C. (1998) Purification and *in vitro* reconstitution of the essential protein components of an aromatic polyketide synthase. *Biochemistry* 37,2084-2088.
- Cortés, J., Wiesmann, K.E.H., Roberts, G.A., Brown, M.J.B., Staunton, J. and Leadlay, P.F. (1995) Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage. *Science*, 268, 1487-1489.

30

ART 34 AMET

-42-

Donadio, S., McAlpine, J.B., Sheldon, P.J., Jackson, M. and Katz, L. (1993)
Proc. Natl. Acad. Sci. USA, **90**, 7119-7123.

Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J. and Katz, L.
5 (1991) Modular organization of genes required for complex polyketide
biosynthesis. *Science*, **252**, 675-679

Elsner, A., Engert, H., Saenger, W., Hamoen, L., Venema, G. and
Bernhard, F. (1997) Substrate specificity of hybrid molecules from peptide
10 synthetases. *J. Biol. Chem.* **272**, 4814-4819.

Fujimoto, D., Srinivasan, P.R. and Borek, E. (1965) On the nature of the
deoxyribonucleic acid methylases. Biological evidence for the multiple
nature of the enzymes. *Biochemistry* **4**, 2849-2855.

15 Geier, G. E. and Modrich, P. (1979) Recognition sequence of the *dam*
methylase of *Escherichia coli* K12 and mode of cleavage of *Dpn* I
endonuclease. *J. Biol. Chem.*, **254**, 1408-1413.

20 Grabley, S., Granzer, E., Hutter, K., Ludwig, D., Mayer, M., Thiericke, R.,
Till, G., Wink, J., Phillips, S. and Zeeck, A. (1992) *J. Antibiot.* **45**, 56-65.

Jacobsen, J.R., Hutchinson, C.R., Cane, D.E. and Khosla, C. Precursor-
directed biosynthesis of erythromycin analogs by an engineered polyketide
25 synthase. *Science* **277**, 367-369 (1997)

Joshi, A.K. and Smith S. (1993) Construction of a cDNA encoding the
multifunctional animal fatty acid synthase and expression in *Spodoptera*
frugiperda cells using baculoviral vectors. *Biochem J.*, **296**, 143-149.

30

ART 34 AMDT

-43-

- Kao, C.M., Luo, G.L., Katz, L., Cane, D.E. and Khosla, C. (1995) *J. Am. Chem. Soc.*, **117**, 9105-9106.
- Kao, C.M., Luo, G.L., Katz, L., Cane, D.E. and Khosla, C. (1996) *J. Am. Chem. Soc.*, **118**, 9184-9185.
- Kao, C.M., Luo, G.L., Katz, L., Cane, D.E. and Khosla, C., (1994) *J. Am. Chem. Soc.*, **116**, 11612-11613.
- Kuhstoss, S., Huber, M., Turner, J.R., Paschal, J.W. and Rao, R.N. (1996) *Gene*, **183**, 231-236.
- Lau, J., Fu, H., Cane, D. E. and Khosla, C. (1999) Dissecting the role of Acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units. *Biochemistry*, **38**, 1643-1651.
- MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H. and MacNeil, T. (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61-68.
- Marsden, A.F.A., Wilkinson, B., Cortés, J., Dunster, N.J., Staunton, J. and Leadlay, P.F. (1998) *Science*, **279**, 199-202.
- Marshall, J.A. and Johns, B.A. (1998) Total synthesis of (+)-discodermolide. *J. Org. Chem.* **63**, 7885-7892.
- McDaniel, R. et al. (1999) and references therein. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc. Natl. Acad. Sci. USA*, **96**, 1846-1851.

- Miller, R.V. and Kokjohn, T.A. (1990) General microbiology of *recA*: Environmental and evolutionary significance. *Annu. Rev. Microbiol.*, **44**, 365-394.
- 5 Nußbaumer, B. and Wohlleben, W. (1994) Identification, isolation and sequencing of the *recA* gene of *Streptomyces lividans* TK24. *FEMS Microbiol. lett.*, **118**, 51-56.
- 10 Oliynyk, M., Brown, M.J.B., Cortés, J., Staunton, J. and Leadlay, P.F. (1996) *Chem. Biol.*, **3**, 833-839.
- Paitan, Y., Alon, G., Orr, E., Ron, E.Z., and Rosenberg, E. (1999) The first gene in the biosynthesis of the polyketide antibiotic TA of *Myxococcus*
- 15 *xanthus* codes for a unique PKS module coupled to a peptide synthetase. *J. Mol. Biol.* **286**, 465-474.
- Rowe, C.J., Cortés, J., Gaisser, S., Staunton, J., Leadlay, P.F. (1998) Construction of new vectors for regulated high-level expression in
- 20 actinomycetes. *Gene*, **216**, 215-223.
- Ruan, X., Pereda, A., Stassi, D.L., Zeidner, D., Summers, R.G., Jackson, M., Shivakumar, A., Kakavas, S., Staver, M.J., Donadio, S. and Katz, L. (1997) Acyltransferase domain substitutions in erythromycin polyketide
- 25 synthase yield novel erythromycin derivatives. *J. Bacteriol.* **179**, 6416-6425.
- Shen, B., Du, L., Sanchez, C., Chen, M. and Edwards, D.J. (1999) Bleomycin biosynthesis in *Streptomyces verticillus* ATCC15003: A model of hybrid peptide and polyketide biosynthesis. *Bioorganic Chemistry* **27**, 123-
- 30 129.
- Tapialas, D.M., Roman, M., Fenical, W., Stout, T.J. and Clardy, J. (1991)

ART 34 AMDT

-45-

Octalactin-A and Octalactin-B - cytotoxic 8-membered-ring lactones from a marine bacterium, *Streptomyces* sp. *J. Amer. Chem. Soc.* **113**, 4682-4683.

- TerHaar, E., Kowalski, R.J., Hamel, E., Lin, C.M., Longley, R.E.,
5 Gunasekera, S.P., Rosenkranz, H.S. and Day, B.W. (1996)
Discodermolide, a cytotoxic marine agent that stabilizes microtubules more
potently than taxol. *Biochemistry* **35**, 243-250.

- Yamada, S., Tanaka, A. and Oritani, T. (1995) Total synthesis of
10 Decarestrictine-J. *Biosci. Biotech. & Biochem.* **59**, 1657-1660

Ziemann, R. and Betlach, M.C. (1999) Recombinant Polyketide Synthesis
in *Streptomyces*: Engineering of improved host strains. *BioTechniques* **26**,
106-110.

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CLAIMS

- 5 1. A method of assembling several DNA units in sequence in a DNA construct, which method comprises the steps of
- a) providing each DNA unit with a restriction enzyme recognition sequence at its 5' end and with a recognition sequence for the same
10 restriction enzyme at its 3' end that is combined with a recognition site for a DNA modification enzyme.
- b) providing a starting DNA construct having an accessible restriction site for the same or a compatible restriction enzyme and cleaving
15 the starting DNA construct with such a restriction enzyme,
- c) inserting the desired DNA unit and bringing the ligated product into contact with a DNA modification enzyme such that the restriction site at the 3' end of the inserted DNA unit is abolished
20
- d) cleaving the ligated product at an accessible unmodified recognition site for the same or a compatible restriction enzyme,
- e) repeating steps c) and d) to introduce each desired DNA unit
25 to give a DNA construct containing all the desired units in sequence.
2. The method of claim 1 wherein the DNA modification enzyme is a methylase.
- 30 3. The method of claim 2 wherein the methylase is the *dam* methylase of *Escherichia coli*.

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4. The method of claim 3 which comprises the steps of
- a) providing each DNA unit with an *Xba*I recognition sequence
5 5'X₂TCTAGA3' (where X₂ is not GA) at its 5' end and with an *Xba*I
recognition sequence 5'GATCTAGA3' at its 3' end.
- b) providing a starting DNA construct having an accessible *Xba*I
site and cleaving the starting DNA construct with *Xba*I,
- 10 c) inserting the desired DNA unit and using a resulting ligated
product to transform a dam⁺ strain of *E. coli*,
- d) recovering a resulting plasmid and cleaving the plasmid at an
15 accessible *Xba*I site with *Xba*I,
- e) repeating steps c) and d) to introduce each desired DNA unit
to give a DNA construct containing all the desired units in sequence.
- 20 5. The method of any one of claims 1 to 4, wherein the
recognition sequences for the restriction enzyme and the DNA modification
enzyme are created in the DNA units prior to cutting with the restriction
enzyme.
- 25 6. The method of claim 5 wherein the restriction sites are
created in the fragment by means of a primer extension reaction.
7. The method of any one of claims 1 to 6, wherein the DNA
construct is an expression vector capable of facilitating expression of the
30 protein encoded by the desired DNA units

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8. The method of claim 3 or claim 4, wherein the DNA modification is removed and the restriction site re-established by replicating the ligated product in a dam- strain of *E. coli* by means of a suitable vector.

5 9. A method of making an assembly of several DNA units in sequence which method comprises the steps of:

a) providing a first DNA unit with a recognition sequence for a first restriction enzyme at its 3' end, and cleaving the said first DNA unit
10 with said first restriction enzyme,

b) providing each other DNA unit with a recognition sequence at its 5' end for a second restriction enzyme which has a compatible ligation sequence with that of the first restriction enzyme, and a downstream
15 recognition sequence for said first restriction enzyme followed by a downstream recognition sequence for a third restriction enzyme at its 3' end, and cleaving each said other DNA unit with the second and third restriction enzymes,

20 c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product such that the ligation of the two units abolishes the recognition site for the first restriction enzyme at the ligation junction, and cleaving the ligated product with said first restriction enzyme,

25 d) ligating the product from c) with a desired DNA unit from b) to form a ligated product and cleaving the ligated product with said first restriction enzyme

e) repeating step d) with each other DNA unit in turn so as to
30 assemble the DNA units in sequence.

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10. The method of claim 9 which method comprises the steps of:

a) providing a first DNA unit with an *Xba*I recognition sequence 5'TCTAGA3' at its 3' end, and cleaving the said first DNA unit with *Xba*I,

5

b) providing each other DNA unit with a *Spe*I recognition sequence 5'ACTAGT3' at its 5' end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3' end, cleaving each said other DNA unit with *Spe*I and *Sma*I, and dephosphorylating the 5' end of the cleaved DNA unit,

10

c) ligating the said first DNA unit with a desired other DNA unit to form a ligated product and cleaving the ligated product with *Xba*I,

15

d) ligating the product from c) with a desired DNA unit from b) to form a ligated product and cleaving the ligated product with *Xba*I

e) repeating step d) with each other DNA unit in turn so as to assemble the DNA units in sequence.

20

11. The method of claim 9 or claim 10 wherein the assembly occurs via stepwise addition of fragments to a vector

25 12. The method of claim 9 or claim 10 wherein the said first DNA unit is attached to the solid phase for use in step c)

13. The method of claim 12, wherein the solid phase is split and mixed between steps c), d), and e) to make several different assemblies.

30

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14. The method of any one of claims 9-13, wherein the recognition sequences in one or more of the DNA units are introduced by means of extension primers.

5 15. The method of any one of claims 9-14 wherein the assembly of several DNA units is inserted in to an expression vector which is used to transform a host capable of expressing the protein encoded by the vector

10 16. The method of any one of claims 1-15, wherein one or more of the DNA units encodes a catalytic or transport protein domain. (*see Kleinkauf peptide/polyketide systems paper*)

15 17. The method of claim 16 wherein one or more of the DNA units are derived from polyketide synthesising enzyme domain DNA sequences.

18. The method of claim 16 wherein one or more of the DNA units are derived from peptide synthesising enzyme domain DNA sequences.

20 19. The method of claim 16 wherein one or more of the DNA units are derived from hybrid peptide polyketide enzyme domain DNA sequences.

25 20. The method of claim 16 wherein one or more of the DNA units are derived from fatty acid synthesising enzyme domain DNA sequences

30 21. The method of claim 16 wherein one or more of the DNA units encode modules comprising one or more catalytic or transport domains

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22. DNA constructs incorporating one or more DNA assemblies encoding synthetic enzymes made by any one of the methods of claims 1-21.
- 5
23. Synthetic enzymes encoded by one or more DNA assemblies made by the methods of anyone of claims 1-21
24. Hosts expressing DNA constructs encoding one or more synthetic enzymes made by any one of the methods of claims 1-21.
- 10
25. Hybrids of transformed hosts expressing one or more DNA constructs encoding synthetic enzymes incorporating a DNA assembly made by any one of the methods of claims 1-21.
- 15
26. Compounds produced by synthetic enzymes encoded by DNA assemblies made by any one of the methods of claims 1-21.
27. A method of synthesising a target molecule comprising the steps of
- 20
- a) examining the composition and stereochemistry of a target molecule,
- 25
- b) determining which catalytic and transport domains need to be present in a synthetic enzyme in order to catalyse the synthesis of the target molecule,
- c) using any one of the methods of claims 1-21 to assemble the required DNA units encoding the catalytic and transport domains into a
- 30

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DNA assembly that encodes said synthetic enzyme which is capable of synthesising the target molecule.

- 5 d) placing the DNA assembly into a vector to allow expression of the synthetic enzyme in a host capable of synthesising the target molecule after transformation with said vector.

28. The method of claim 27 wherein the transformed host is tested for the presence of the target molecule after step d).

10

29. The transformed host of claim 27.

30. Use of transformed host of claim 27 to produce said target molecule.

15

31. A method of making a synthetic enzyme to catalyse the synthesis of a target molecule comprising the steps of

20 a) examining the composition and stereochemistry of a target molecule,

b) determining which catalytic and transport domains need to be present in the synthetic enzyme in order to catalyse the synthesis of the target molecule,

25

c) using any one of the methods of claims 1-21 to assemble the required DNA units encoding the catalytic and transport domains into a DNA assembly that encodes an enzyme which is capable of synthesising the target molecule.

30

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d) expressing the DNA assembly in a suitable host to produce the enzyme.

32. A library of DNA units encoding catalytic or transport protein domains, wherein each DNA unit has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

33. The library of claim 32, wherein each DNA unit has a *XbaI* recognition sequence 5'X₁X₂TCTAGA3' (where X₁X₂ is not GA) at its 5'-end and an *XbaI* recognition sequence 5'GATCTAGA3' at its 3'-end.

34. A library of DNA units encoding catalytic or transport protein domains, wherein each DNA unit has a recognition sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed by a downstream recognition sequence for a third restriction enzyme at its 3' end, such that the DNA units, once restricted by the first and second restriction enzymes can be ligated together to abolish the restriction sites at the ligation junction.

35. The library of claim 34, wherein each DNA unit has a *SpeI* recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *XbaI* recognition sequence 5'TCTAGA3' followed by a downstream *SmaI* recognition sequence 5'CCCGGG3' at its 3'-end.

36. The library of claim 32 or claim 35, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

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37. A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence for a restriction enzyme at its 5'-end and a second recognition sequence for the same or a compatible enzyme at its 3'-end which incorporates a recognition sequence for a DNA modifying enzyme.

38. The module as claimed in claim 37, wherein the module has an *Xba*I recognition sequence 5'XXTCTAGA3' (where XX is not GA) at its 5'-end and an *Xba*I recognition sequence 5'GATCTAGA3' at its 3'-end.

39. A module comprising a DNA sequence encoding a functional set of polyketide synthetic domains wherein the module has a recognition sequence at its 5' end for a first restriction enzyme, and a downstream recognition sequence for a second restriction enzyme followed a downstream recognition sequence for a third restriction enzyme at its 3' end, such that the DNA units, once restricted by the first and second restriction enzymes can be ligated together to abolish the restriction sites at the ligation junction.

40. The module as claimed in claim 39, wherein the module has a *Spe*I recognition sequence 5'ACTAGT3' at its 5'-end, and a downstream *Xba*I recognition sequence 5'TCTAGA3' followed by a downstream *Sma*I recognition sequence 5'CCCGGG3' at its 3'-end.

41. A module as claimed in claim 37 or claim 39, wherein the DNA units encode polyketide synthetic domains, comprising two KS domains, at least two AT domains, two KR domains, two DH domains, two ER domains, an ACP domain and a TE domain.

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42. A vector containing one or more modules as claimed in claim 37 or claim 39.

43. The vector as claimed in claim 42, wherein a non-functional *recA* gene is also present.

44. A method of transforming a host with one or more synthetic DNA assemblies encoding enzyme domains which comprises the steps of:

- a) Inserting said DNA assembly into a vector containing a mutated internal fragment of a *recA* gene sequence such that the vector is capable of undergoing homologous recombination with the *recA* gene of the host,
- b) bringing said vector into contact with a host chromosome under conditions which permit homologous recombination to take place,
- c) disrupting the host *recA* gene by the integration of the DNA of said vector into the chromosome.

45. The method of claim 44 wherein the expression vector is used to transform a *Streptomyces* host.

46. The method of claim 44 or claim 45, wherein the DNA assemblies are modules according to claim 37 or claim 39.

47. A host lacking a *recA* function, transformed with a vector containing one or more modules according to claim 37 to 39.

48. A kit containing DNA units, DNA modules, vectors, DNA manipulation hosts, DNA modification hosts, expression hosts, or solid phase elements for use in the methods claimed herein.

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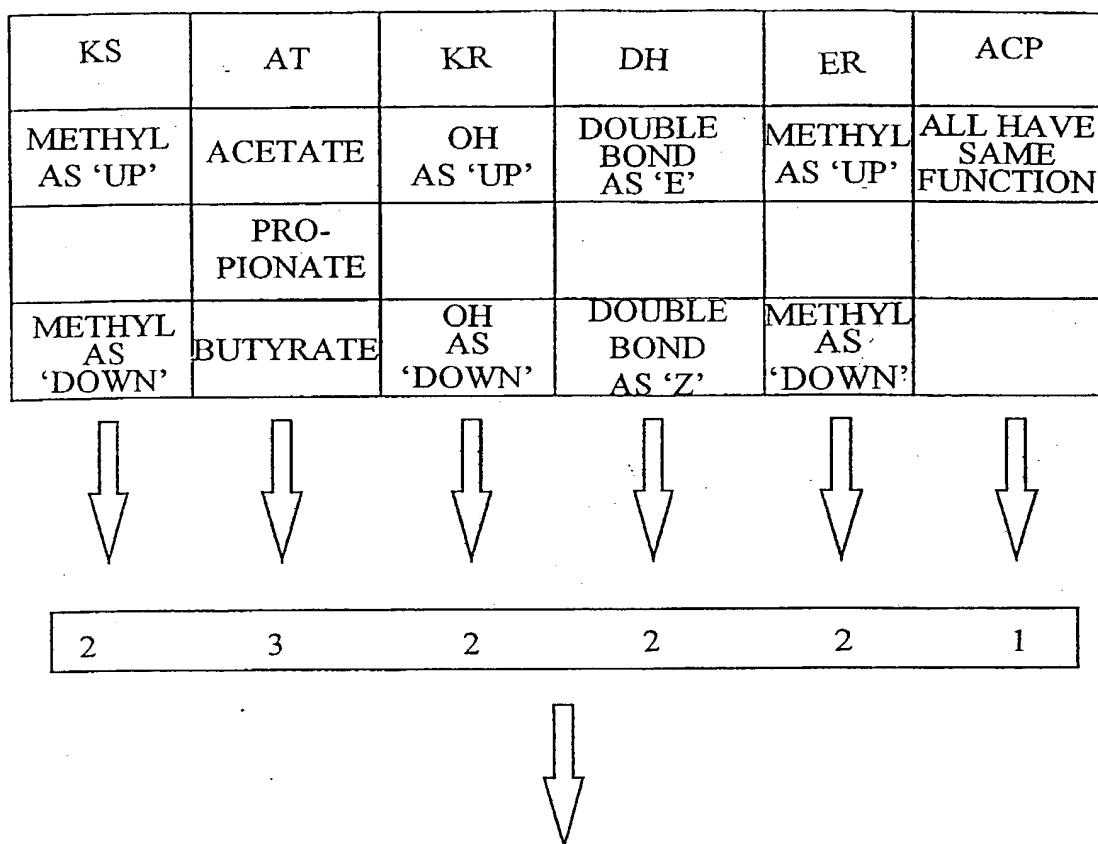


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(54) Title: DNA MANIPULATION METHODS AND APPLICATIONS FOR SYNTHETIC ENZYMES

(57) Abstract: The invention comprises a method of assembling several DNA units in sequence in a DNA construct and all derivatives of this method. In particular the production of synthetic enzymes is contemplated. Each DNA unit is provided with the same restriction enzyme recognition site at its 5' and 3' ends. The restriction recognition site at its 3' end being combined with a recognition site for a DNA modification enzyme. A DNA construct having the same or a compatible accessible restriction site, as provided in the DNA unit, is cleaved at the restriction site by the appropriate restriction enzyme. The desired DNA unit is then inserted into the DNA construct, this ligated product subsequently being brought into contact with a DNA modification enzyme such that the restriction site at the 3' end of the inserted DNA unit is abolished. The ligated product is then cleaved at the remaining unmodified restriction recognition site and a subsequent DNA unit is inserted. This process is repeated introducing each desired DNA unit to give a DNA construct containing all the desired units in sequence.

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TOTAL NUMBER OF DOMAINS
REQUIRED FOR EVERY CONCEIVEABLE
POLYKETIDE REACTION= 12

FIG. 1.

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STREPTOMYCES HOST

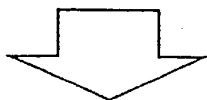
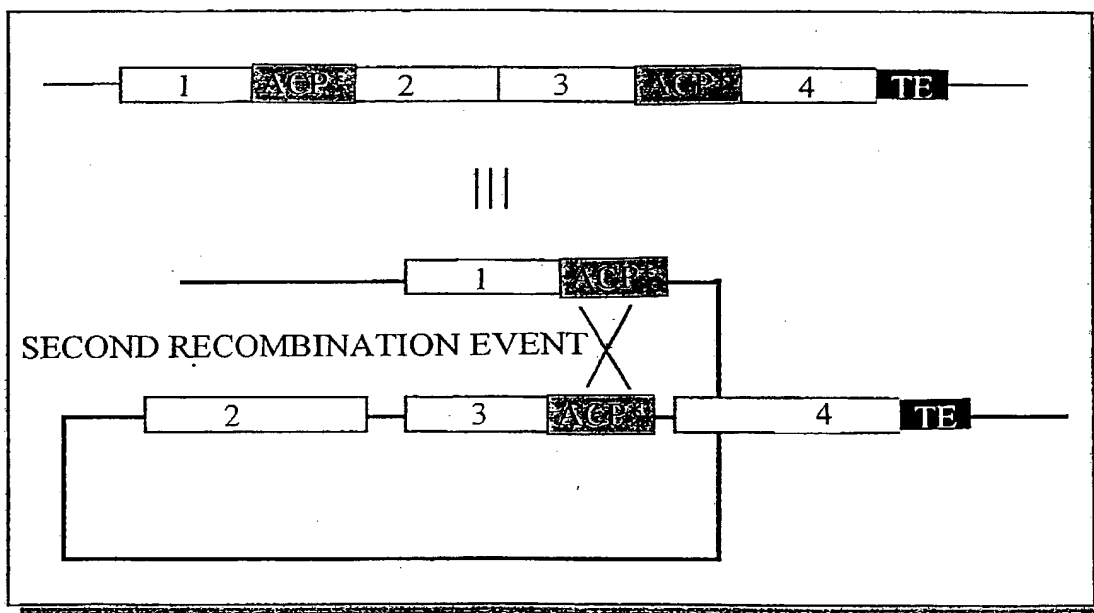
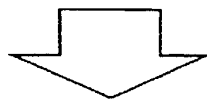
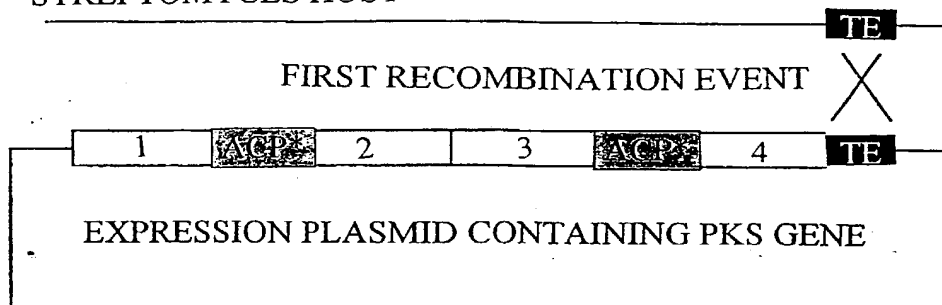
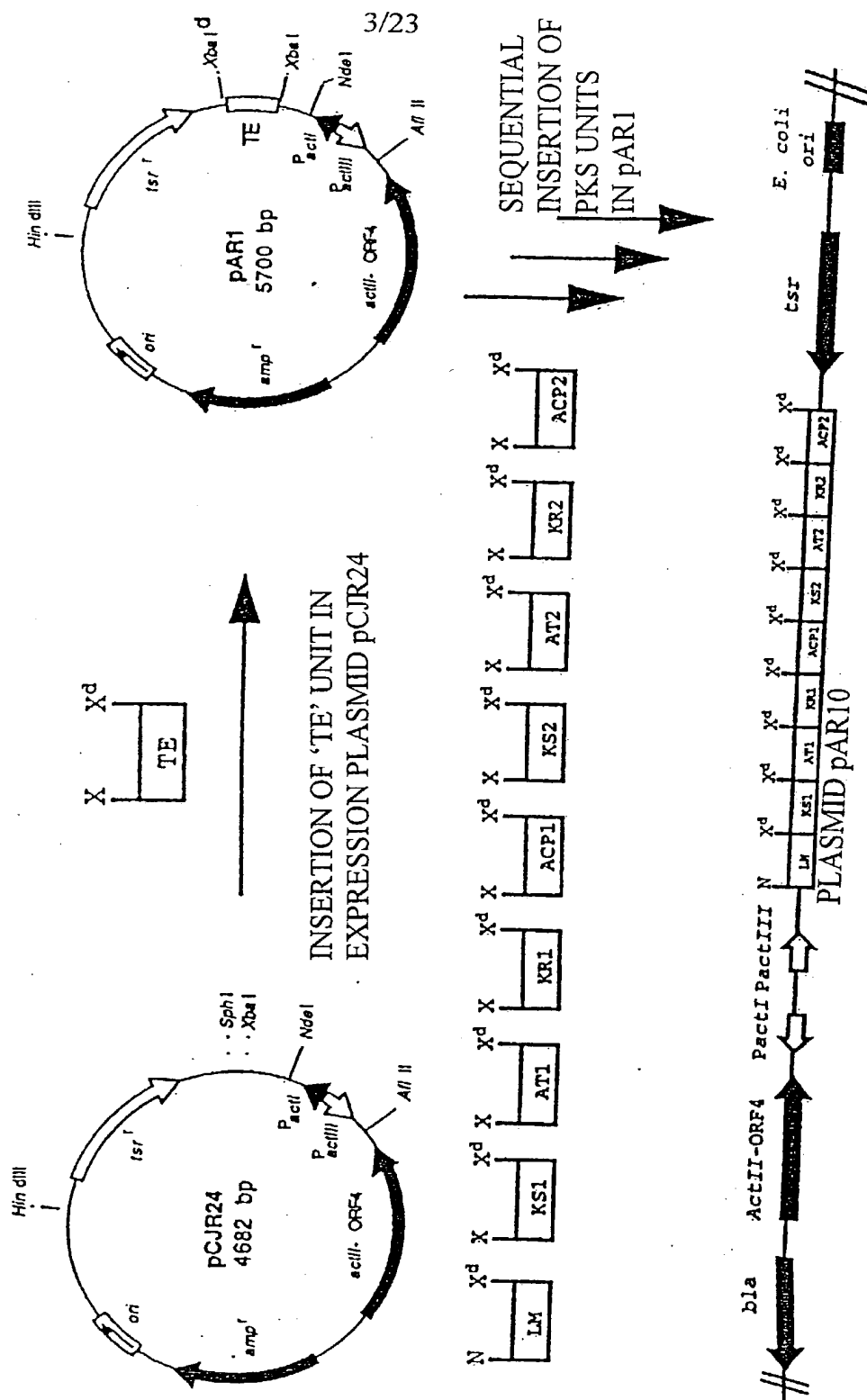


FIG.2.

FIG.3A.



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...
 ↓
 INTEGRATION INTO S.ERYTHRAEL/JC2 STRAIN
 FOLLOWED BY PROTEIN EXPRESSION

Assembled DEBS1-TE

MODULE 1
 MODULE 2
 TE

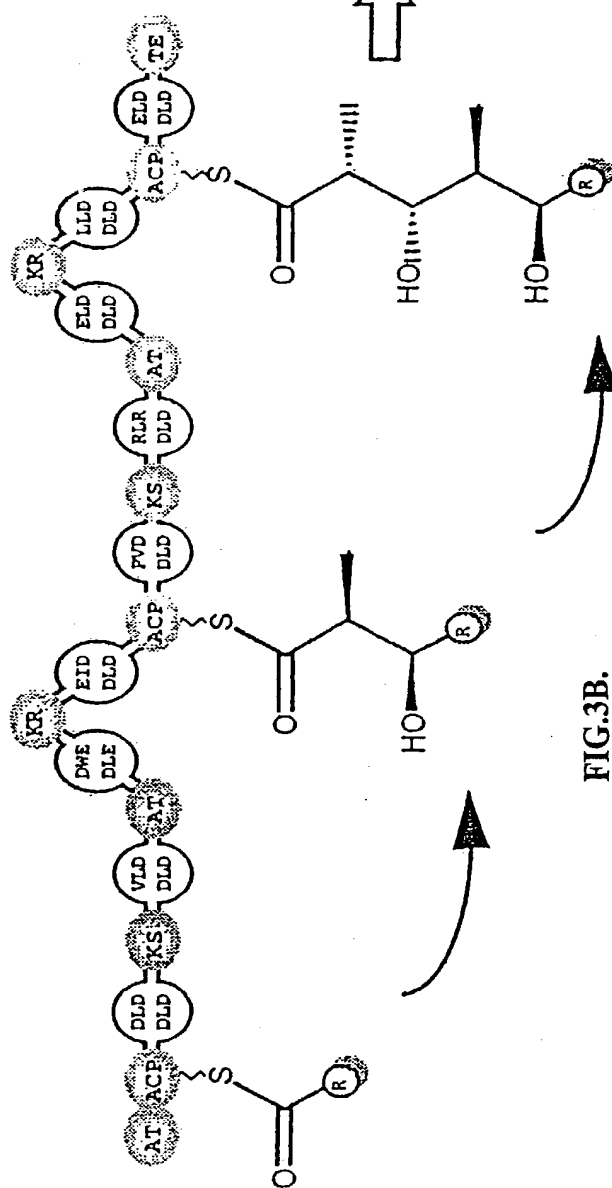
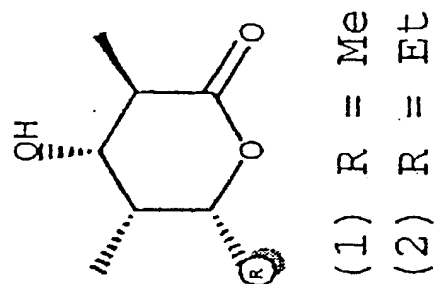


FIG.3B.



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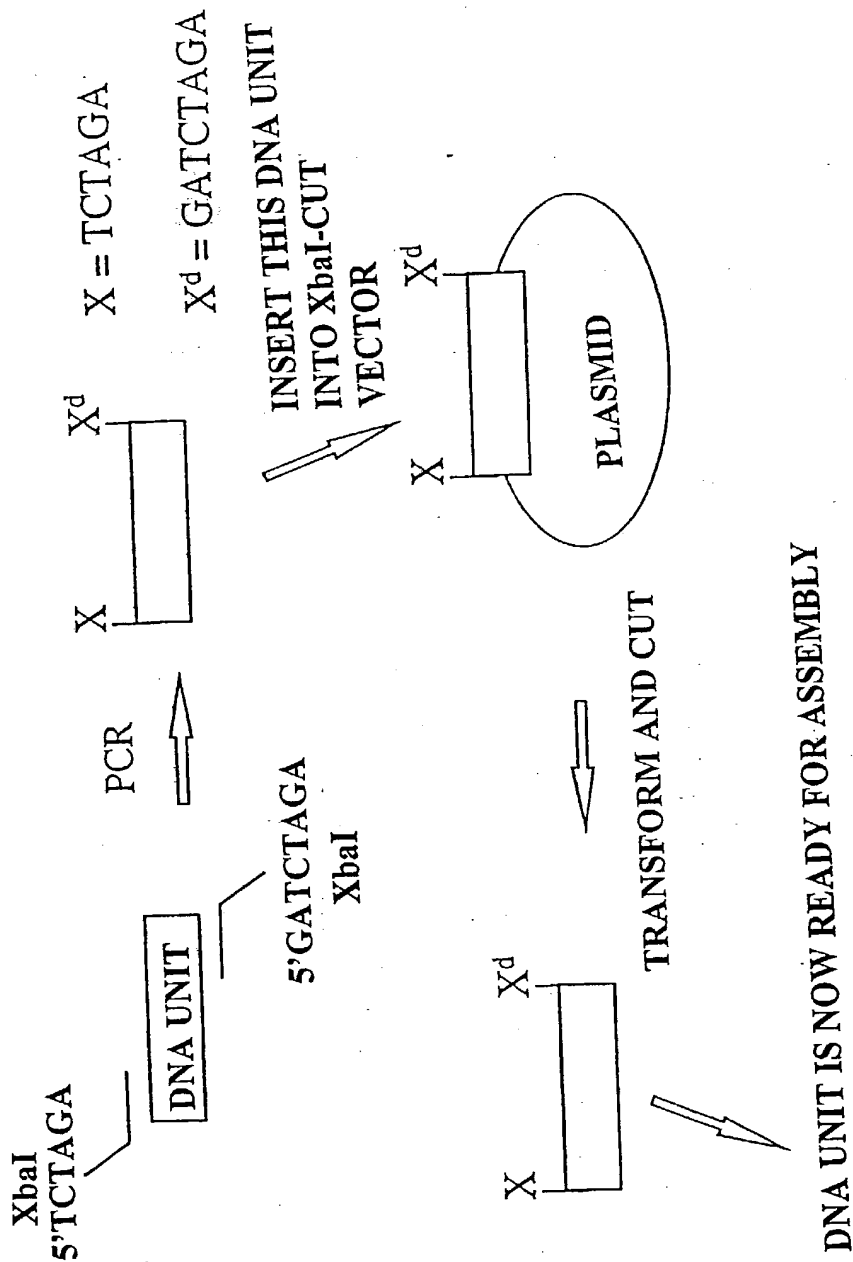


FIG.4.

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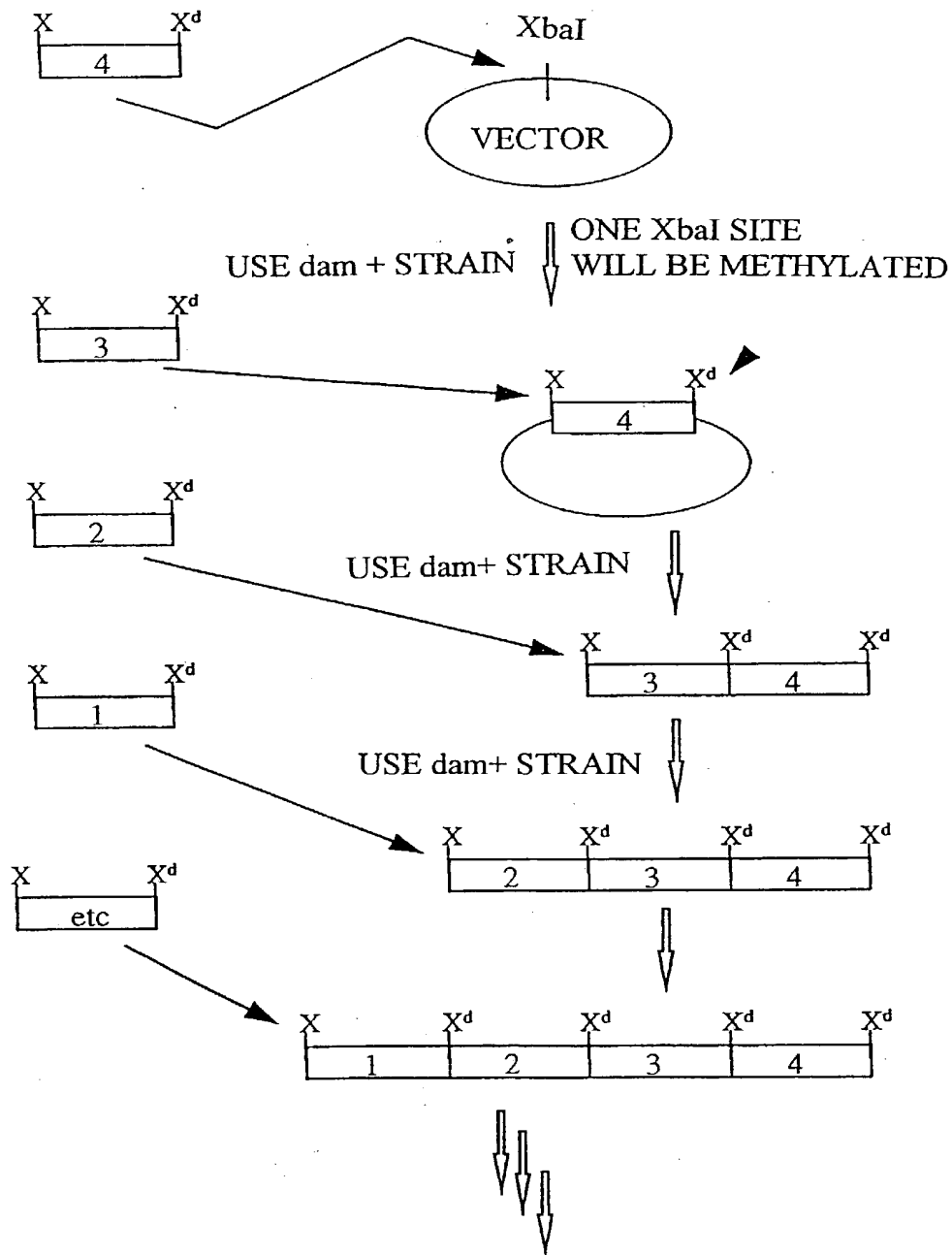


FIG.5.

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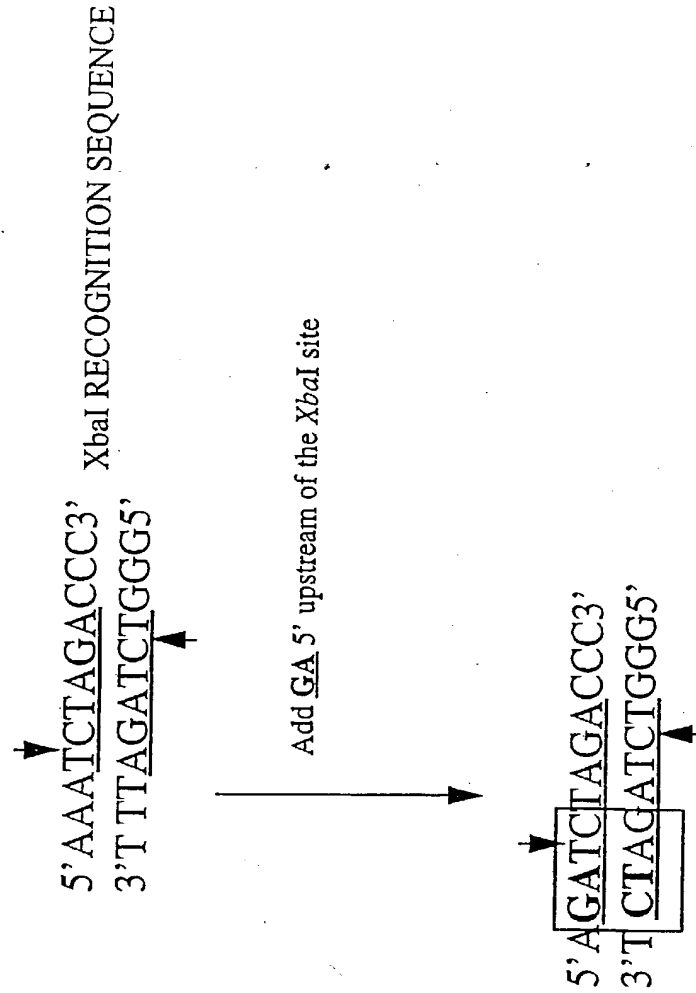


FIG.6.

SUBSTITUTE SHEET (RULE 26)



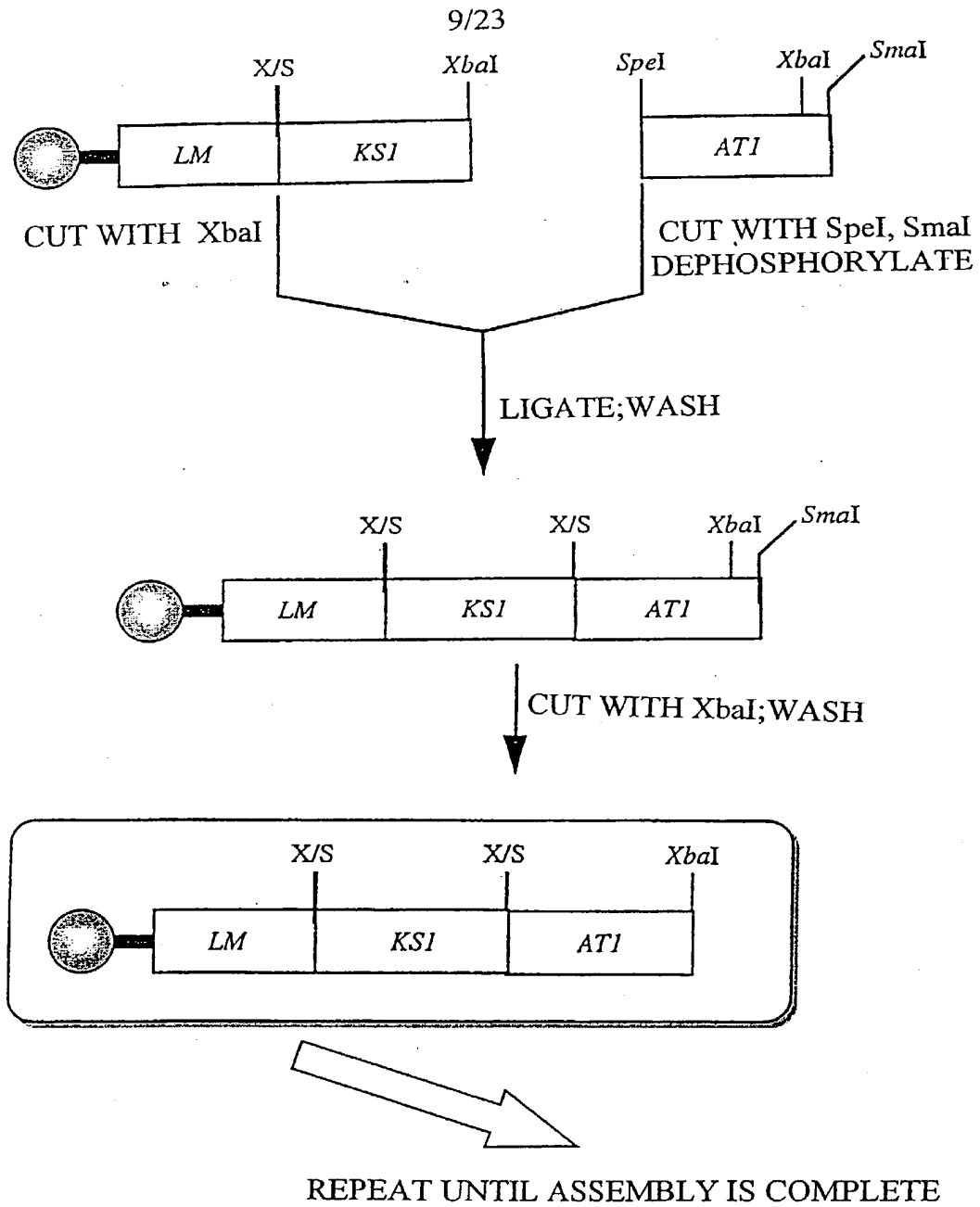
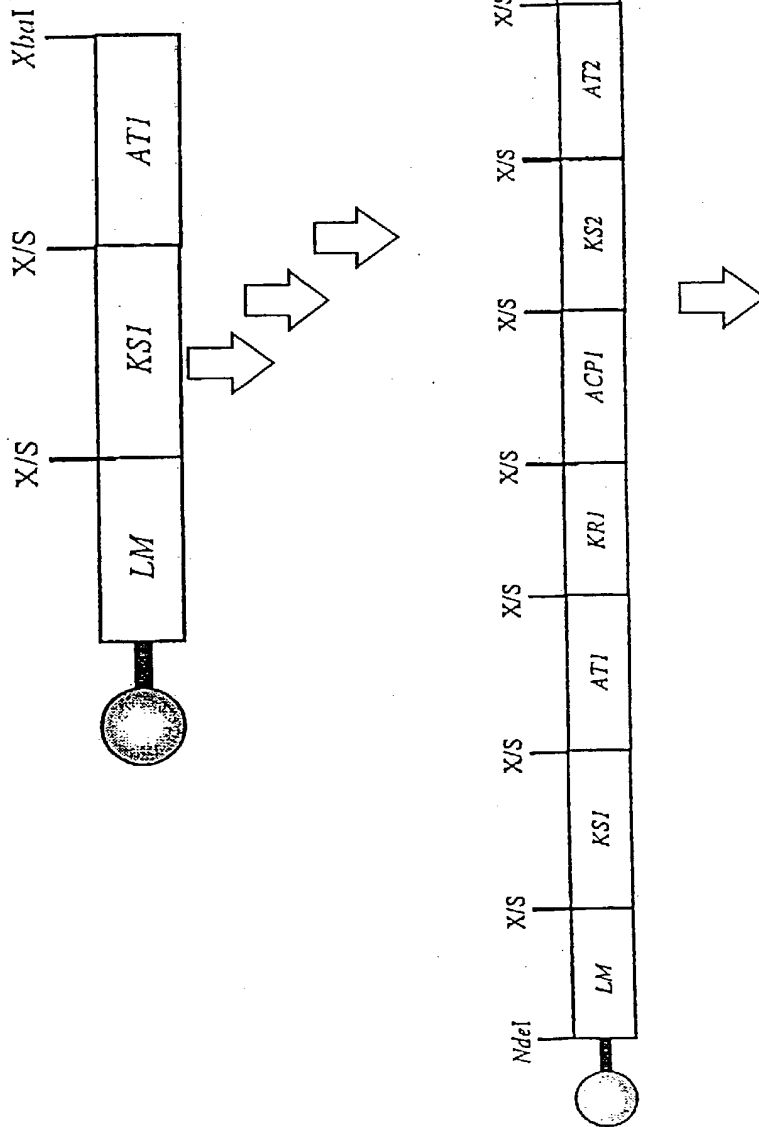


FIG.8.

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CLONE IN pCIR24 AND EXPRESS IN S. ERYTHRAEA

FIG.9.

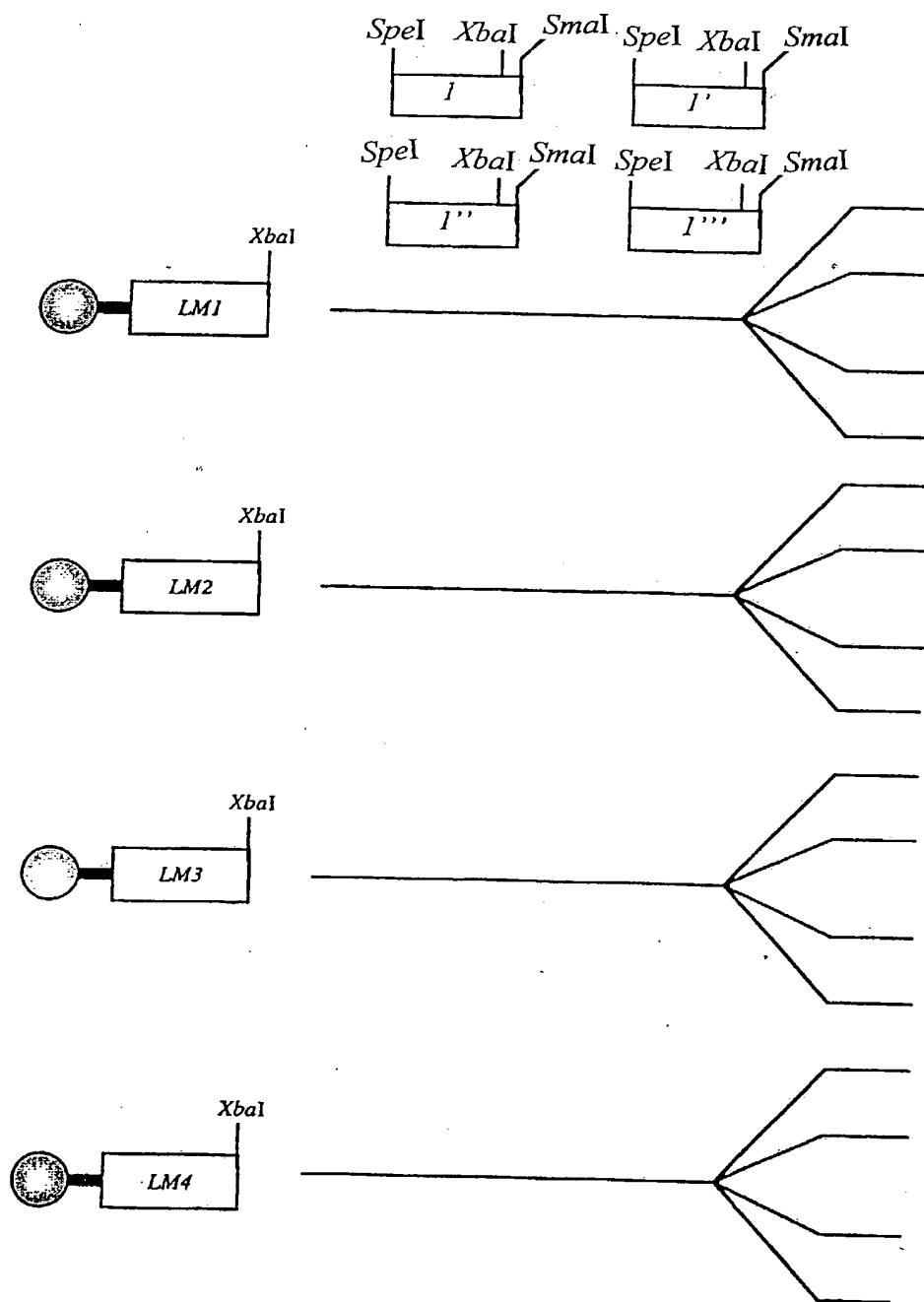


FIG.10.

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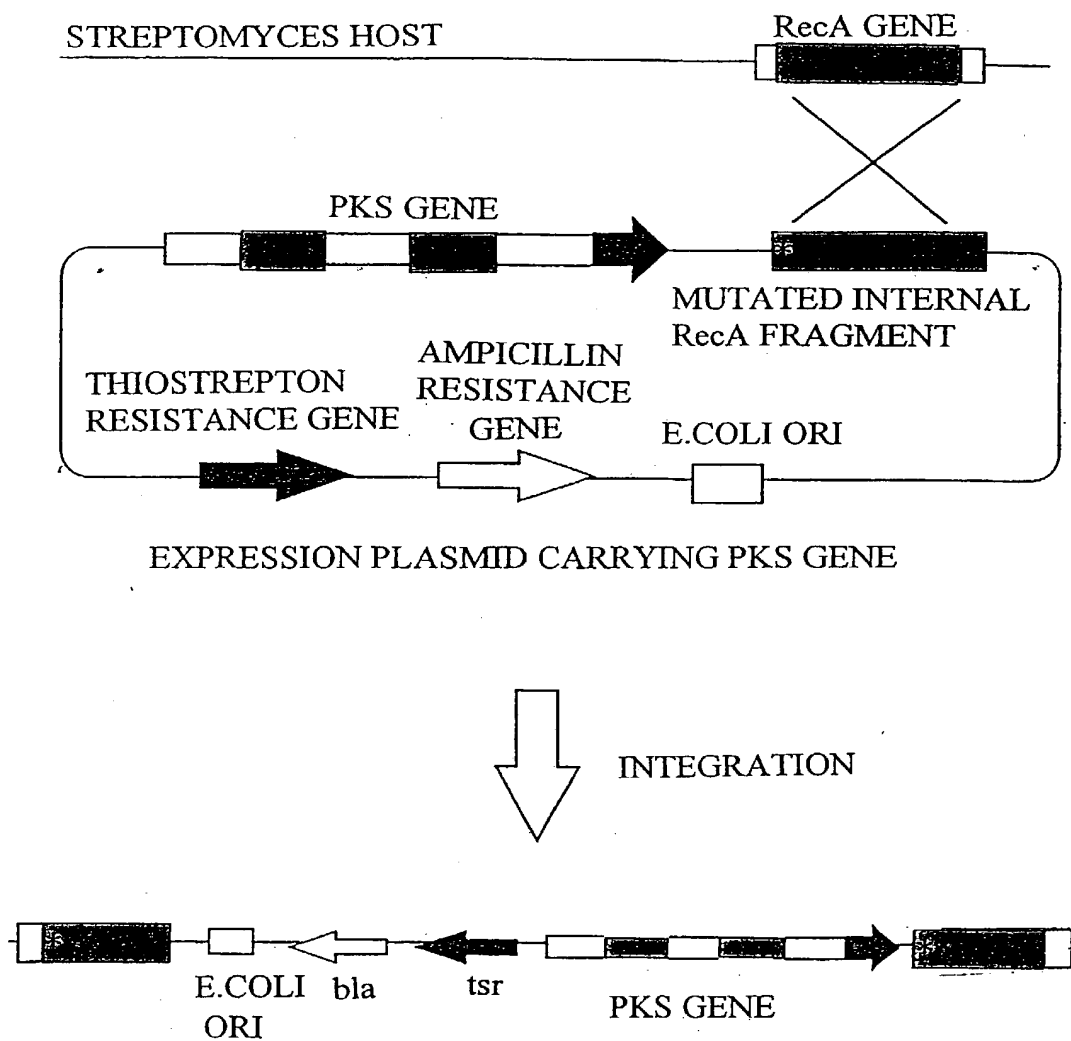
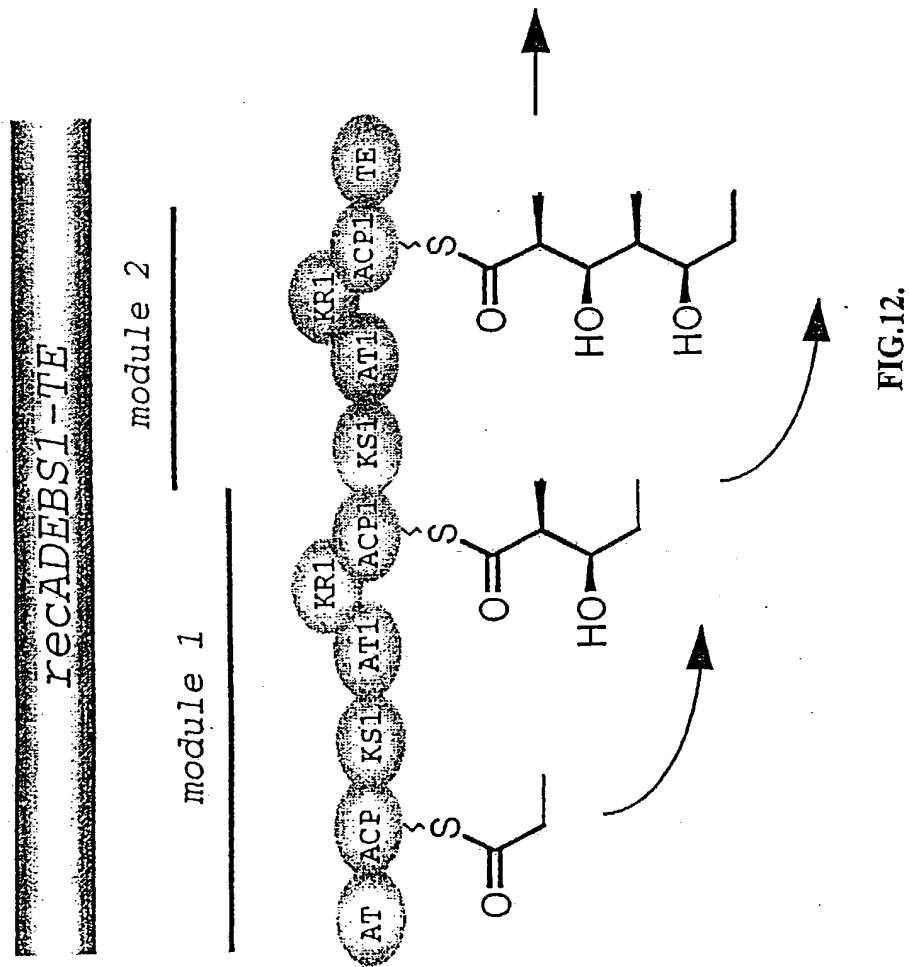
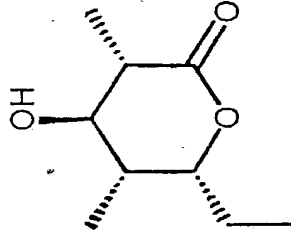


FIG.11.

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S.I.	1	MAGTDREKALDAALAQIERQFGKGAVMRMGDRTPNEPIEVIPTGSTALDVA	50
S.a.	1	MAGTDREKALDAALAQIERQFGKGAVMRMGDRSKEPIEVIPTGSTALDVA	50
	51	LGVGGIPRGRVVEVYGPRESSGKTTTLTHAVANAQKAGGQVAFVDAEHALD	100
		: :	
	51	LGVGGLPRGRVIEVYGPRESSGKTTTLTHAVANAQKAGGQVAFVDAEHALD	100
	101	PEYAKKLGVDDIDNLILSQPDNGEQALEIVDMLVRSGALDLIVIDSVAALV	150
		.	
	101	PEYAQKLGVDDIDNLILSQPDNGEQALEIVDMLVRSGALDLIVIDSVAALV	150
		.	
	151	PRAEIEGEMGDSHVGLQARLMSQALRKITSALNQSKTTAIFINQLREKIG	200
	151	PRAEIEGEMGDSHVGLQARLMSQALRKITSALNQSKTTAIFINQLREKIG	200
	201	VMFGSPETTTGGRALKFYASVRLDIRRIETLKDGTDAVGNRTRVKVVKNK	250
	201	VMFGSPETTTGGRALKFYASVRLDIRRIETLKDGTDAVGNRTRVKVVKNK	250
	251	VAPPFKQAEFDILYGQGISREGGLIDMGVENGFVRKAGAWTYEGDQLGQ	300
	251	VAPPFKQAEFDILYGQGISREGGLIDMGVEHGFVRKAGAWTYEGDQLGQ	300
	301	GKENARNFLKDNPDLANEIEKKIKQKLGVGVBHPEE.SATEPGADAASAAP	349
		:	
	301	GKENARNFLKDNPDLANEIEKKIKEKLGVGVRPEEPTATESGPDAAT...	347
	350	ADAAPAVPAPTAKATKSKAAAAKS	374
		:	
	348	AESAPAVPAPATAKVTKAKAAAAKS	372

FIG.13.

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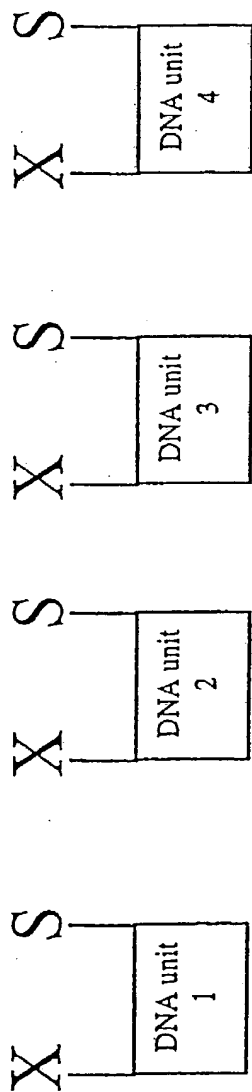
S.l. 1 ATGGCAGGAACCGACCGCGAGAAGGCCCTGGACGCCGCGCTCGCACAGAT 50
|||||
S.a. 1 ATGGCAGGAACCGACCGCGAGAAGGCTCTTGACGCCGCACTCGCACAGAT 50
|||||
51 TGAACGGCAATTTCGGCAAGGGCGCGGTCATGCGCATGGGTGACCGGACCA 100
|||||
51 TGAACGGCAGTTTCGGCAAGGGCGCGGTCATGCGCATGGGCGACCGGTCGA 100
|||||
101 ACGAGCCCATCGAGGTCATCCCGACCGGGTCTACCGCGCTCGACGTGGCC 150
|||||
101 AGGAGCCCATCGAGGTCATCCCGACCGGGTCTGACCGCGCTCGACGTGGCC 150
|||||
151 CTCGGCGTCGGAGGCATCCCGCGTGGCCGTGTCGTGGAGGTCTACGGCCC 200
|||||
151 CTCGGCGTCGGCGGCCTGCCGCGCGGCCGCGTCATCGAGGTCTACGGTCC 200
|||||
201 CGAGTCCTCGGGCAAGACGACCCTGACCCTGCACGCGGTGGCGAACGCGC 250
|||||
201 GGAGTCCTCCGGTAAGACGACCCTGACCCTGCACGCGGTGGCGAACGCGC 250
|||||
251 AGAAGGCCGGCGGCCAGGTCGCGTTTCGTGGACGCCGAGCACGCCCTCGAC 300
|||||
251 AGAAGGCCGGCGGCCAGGTTGGCGTTTCGTGGACGCCGAGCACGCCCTCGAC 300
|||||
301 CCCGAGTACGCGAAGAAGCTCGGTGTCGACATCGACAACCTGATCCTGTC 350
|||||
301 CCCGAGTACGCCAGGAAGCTCGGCGTCGACATCGACAACCTGATCCTGTC 350
|||||
351 CCAGCCGGACAACGGTGAGCAGGCCCTGGAGATCGTGGACATGCTGGTCC 400
|||||
351 CCAGCCGGACAACGGTGAGCAGGCCCTGGAGATCGTGGACATGCTGGTCC 400
|||||
401 GTCCTGGCGCCCTCGACCTCATCGTCATCGACTCCGTCGCCGCGCTCGTC 450
|||||
401 GTCCTGGCGCCCTCGACCTCATCGTCATCGACTCCGTCGCCGCGCTCGTC 450
|||||
451 CCGCGCGCGGAGATCGAGGGCGAGATGGGCGACAGCCACGTGGTCTGCA 500
|||||
451 CCGCGCGCGGAGATCGAGGGCGAGATGGGTGACAGCCACGTGGTCTCCA 500
|||||
501 GGCCCGGCTGATGAGCCAGGCCCTGCGGAAGATCACCAGCGCGCTCAACC 550
|||||
501 GGCCCGGCTGATGAGCCAGGCCCTGCGGAAGATCACCAGCGCGCTCAACC 550
|||||

FIG.14.A.

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S.l. 551 AGTCCAAGACCACCGCGATCTTCATCAACCAGCTCCGCGAGAAGATCGGC 600
|||||
S.a. 551 AGTCCAAGACCACCGCGATCTTCATCAACCAGCTCCGCGAGAAGATCGGC 600
601 GTGATGTTCTGGCTCCCGGAGACCACGACCGGTGGCCGGGCACTGAAGTT 650
|| |||||
601 GTCATGTTCTGGCTCCCGGAGACCACGACCGGTGGCCGGGCGCTCAAGTT 650
651 CTACGCCTCGGTGCGACTCGACATCCGGCGTATCGAGACGCTGAAGGACG 700
|||||
651 CTACGCCTCGGTGCGACTCGACATCCGACGCATCGAGACGCTCAAGGACG 700
701 GCACCGACGCGGTCTGGCAACCGCACCCGCGTCAAGGTGGTCAAGAACAAG 750
|||||
701 GCACCGACGCGGTCTGGCAACCGCACGCGCGTCAAGGTCTCAAGAACAAG 750
751 GTCGCGCCGCCCTTCAAGCAGGCCGAGTTCGACATCCTCTACGGCCAGGG 800
|||||
751 GTCGCGCCGCCCTTCAAGCAGGCCGAGTTCGACATCCTCTACGGCCAGGG 800
801 CATCAGCCGCGAGGGCGGTCTGATCGACATGGGCGTGGAGAACGGCTTCG 850
|||||
801 CATCAGCCGCGAGGGCGGTCTGATCGACATGGGCGTGGAGCACGGCTTCG 850
851 TCCGCAAGGCCGCGCCTGGTACACGTACGAGGGCGACAGCTCGGTCAG 900
|||||
851 TCCGCAAGGCCGCGCCTGGTACACGTACGAGGGCGACAGCTCGGCCAG 900
901 GGCAAGGAGAACGCGCGCAACTTCTGAAGGACAACCCCGACCTGGCCAA 950
|||||
901 GGCAAGGAGAACGCGCGCAACTTCTGAAGGACAACCCCGACCTCGCCAA 950
951 CGAGATCGAGAAGAAGATCAAGCAGAAGCTGGGCGTCGGCGTGACCCCCG 1000
|||||
951 CGAGATCGAGAAGAAGATCAAGGAGAAGCTGGGCGTCGGAGTCCGTCCCC 1000
1001 AGGA...GTCGGCCACCGAGCCCGGCGCGGACGCCGCTCCGCCGCCCCG 1047
|||||
1001 AGGAGCCGACGGCCACCGAGTCCGGACCGGA.....CGCCGCGACG 1041
1048 GCCGACGCCGACCGGCGGTGCCCGCACCCACGACCGCCAAGGCCACCAA 1097
|||||
1042 GCCGAATCCGACCGGCGGTGCCCGCGCCCGCGACCGCCAAGGTCACCAA 1091
1098 GTCCAAGGCCGCGGCGAGCCAAGAGCTGA 1125
| |||||
1092 GGCCAAGGCCGCGGCGAGCCAAGAGCTGA 1119

FIG.14.B.



Sequential ligation
Of DNA units

X = *Xba*I
S = *Spe*I

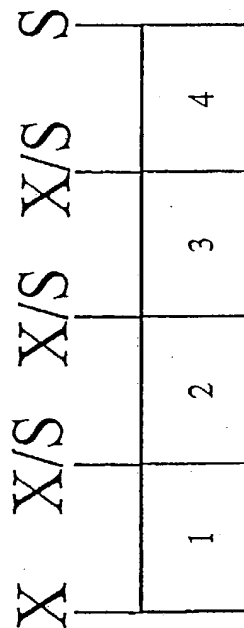


Figure 15.

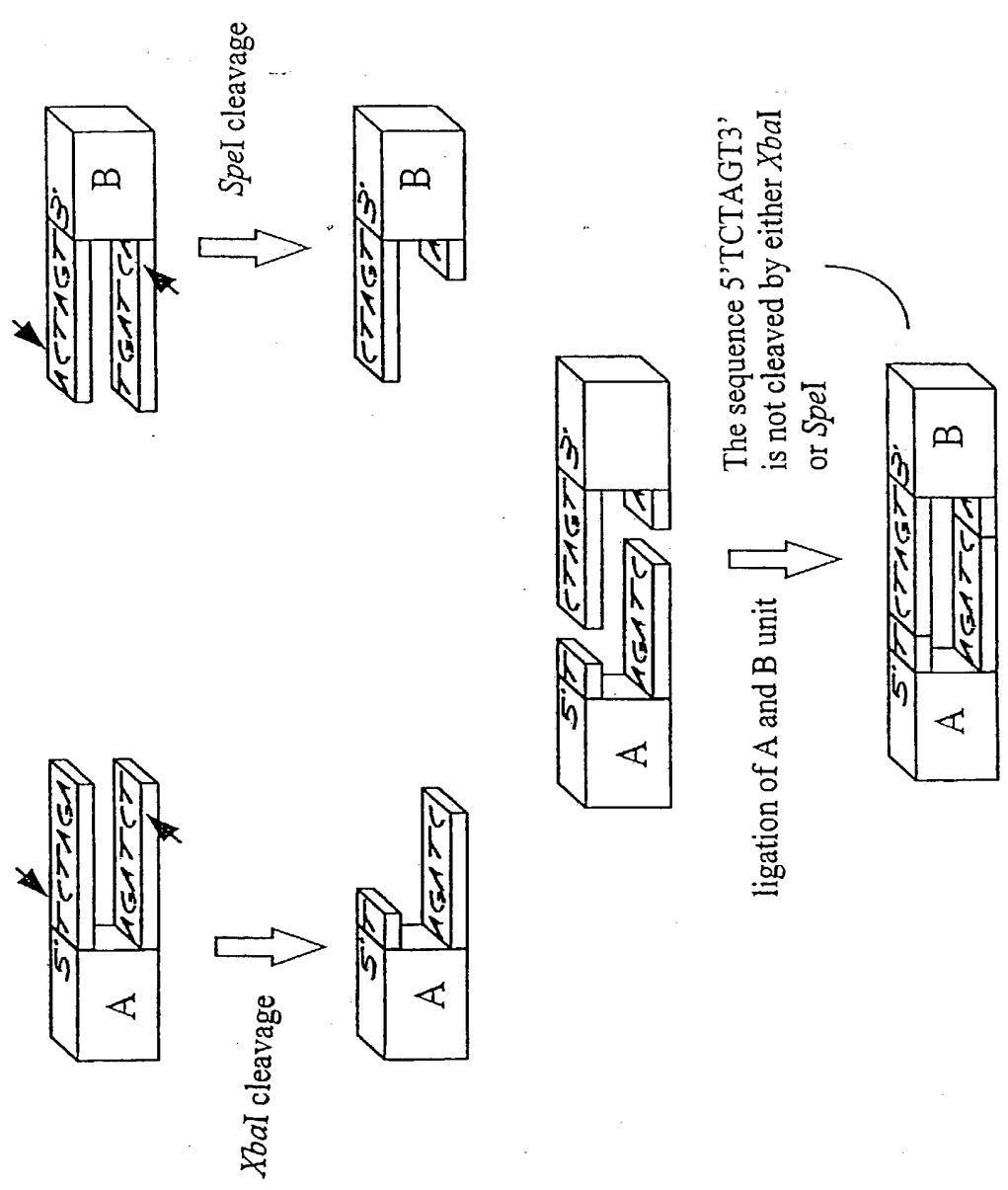


Figure 16.

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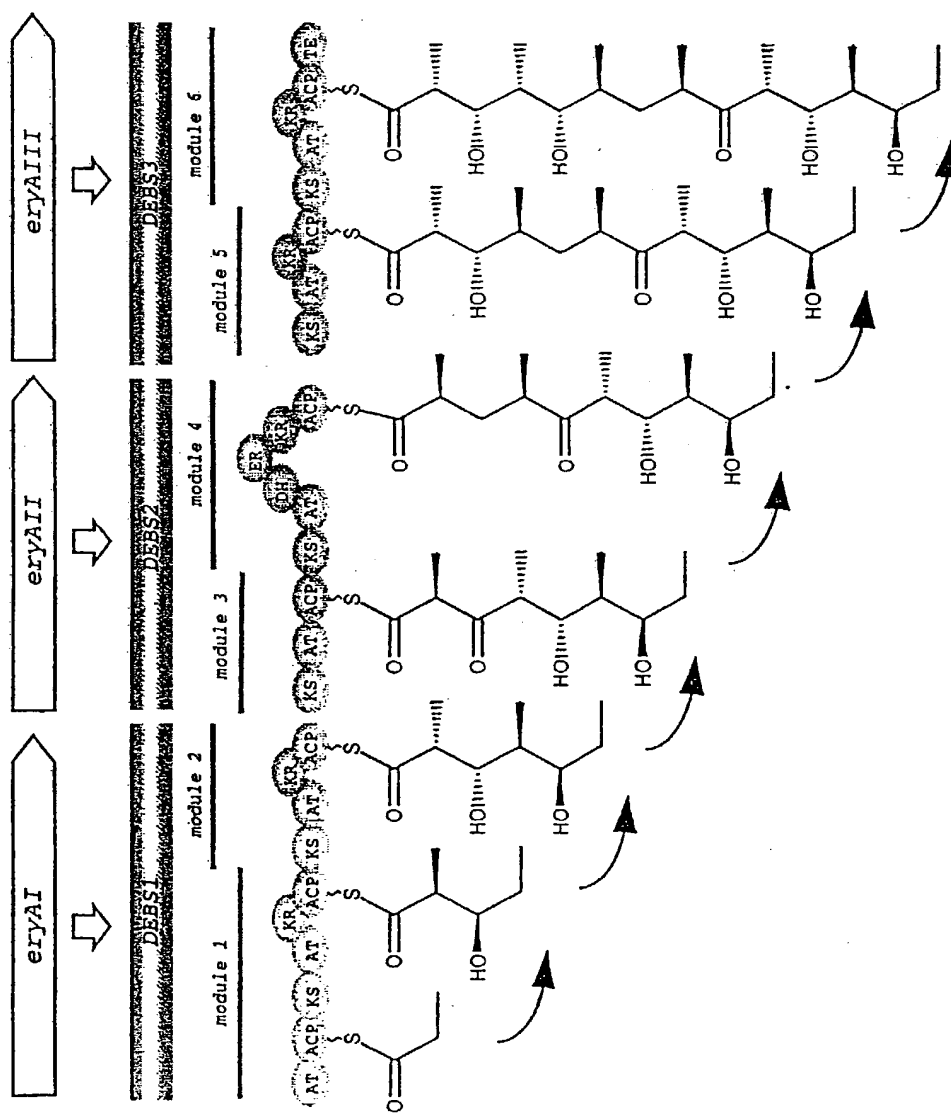
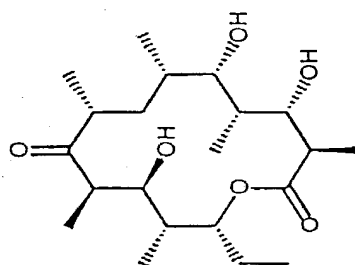


FIG.17.

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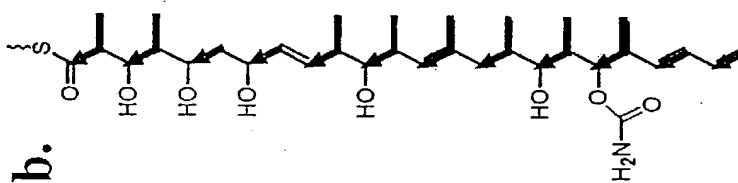
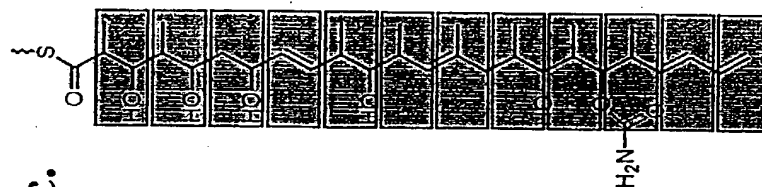
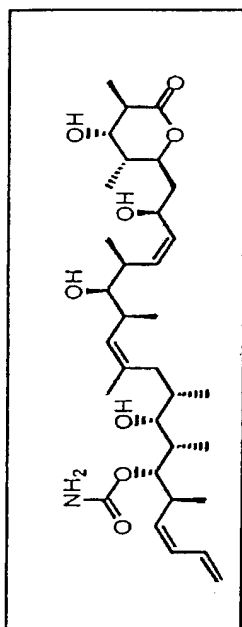
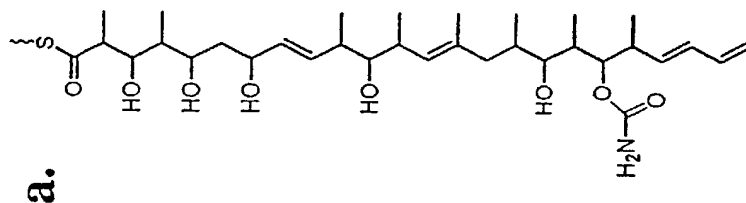
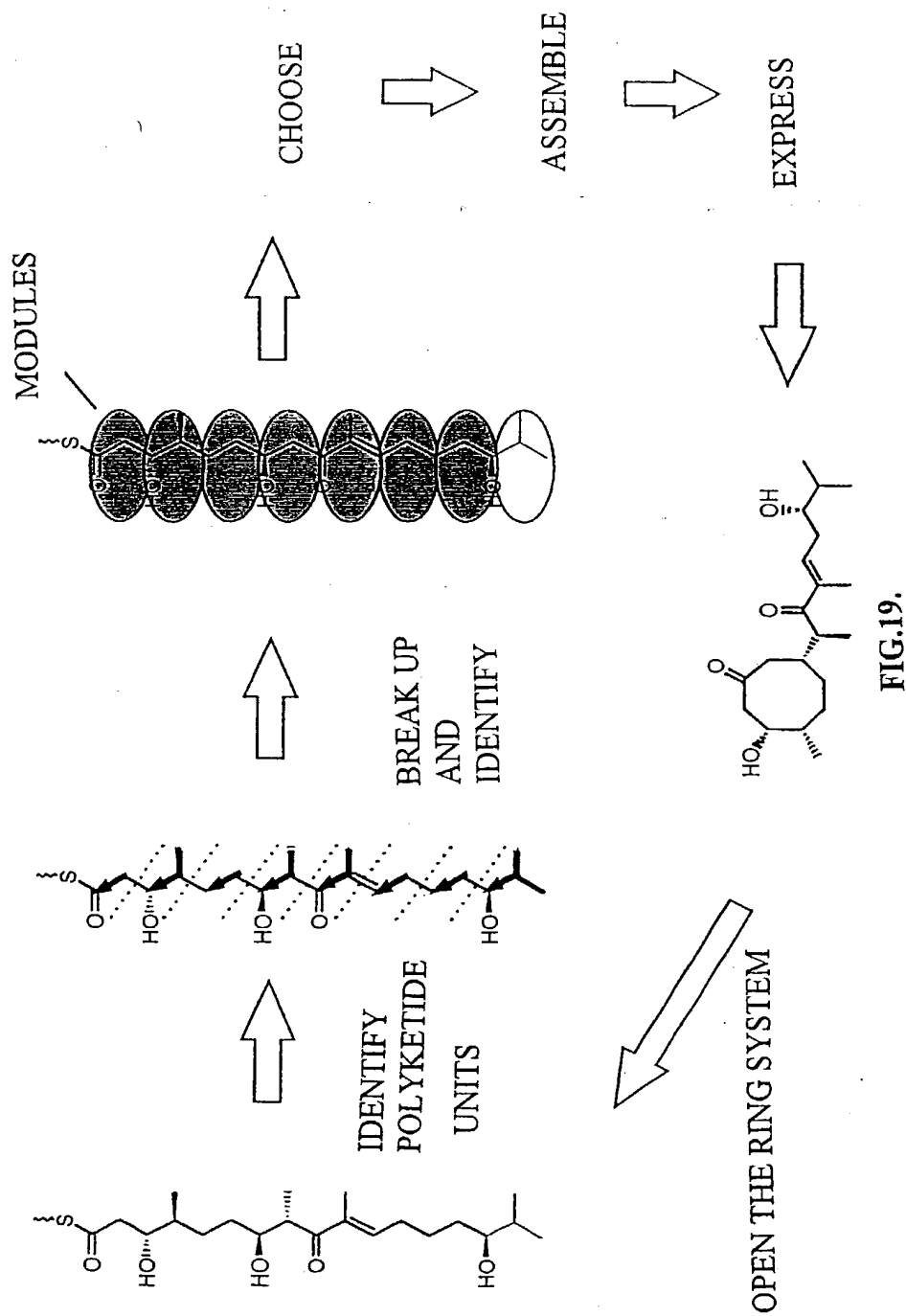


FIG.18.



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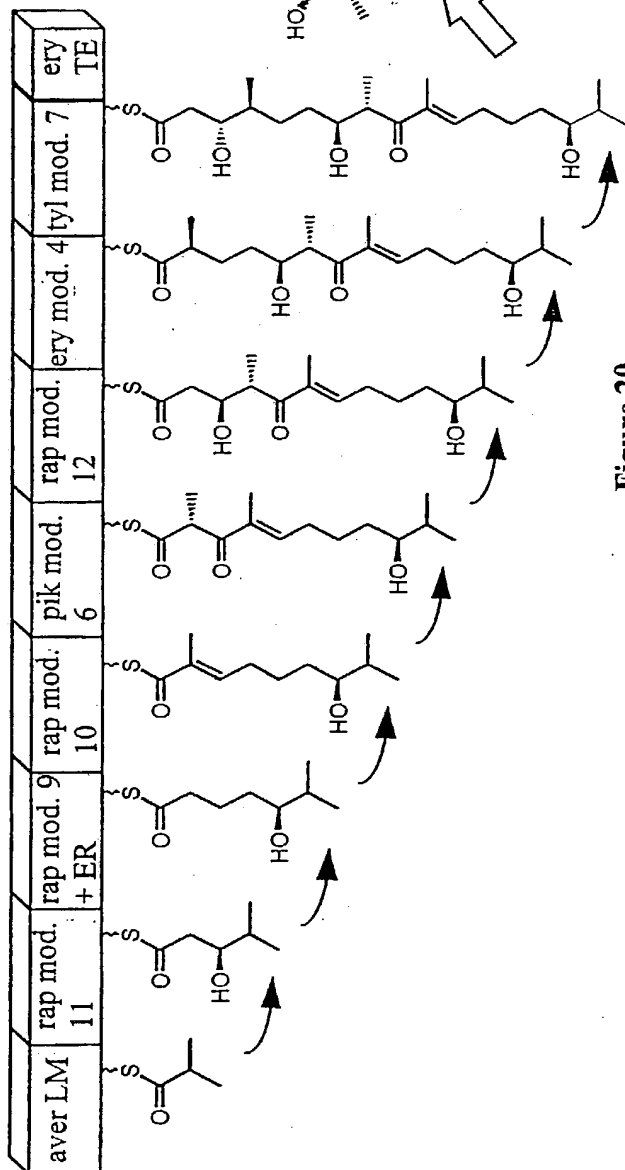
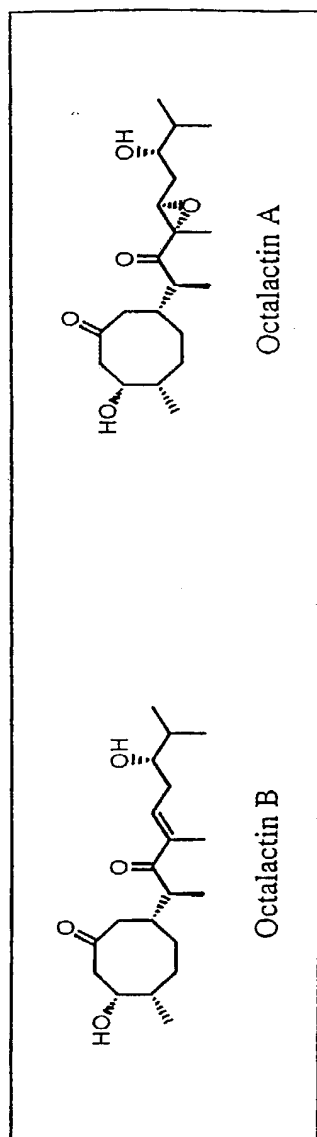
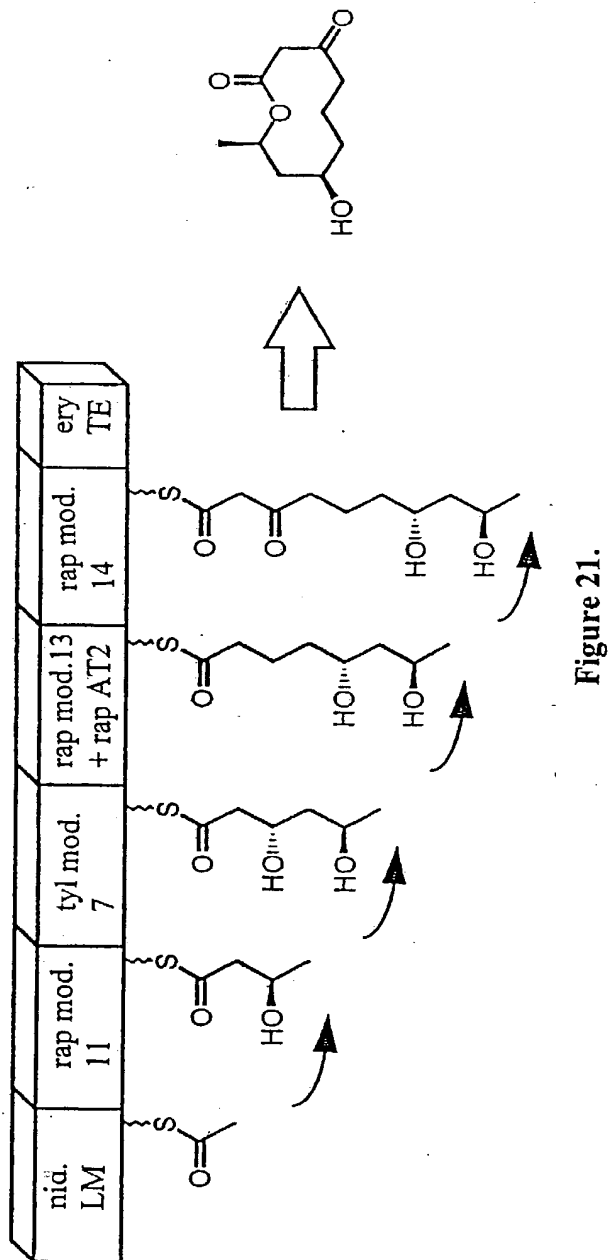
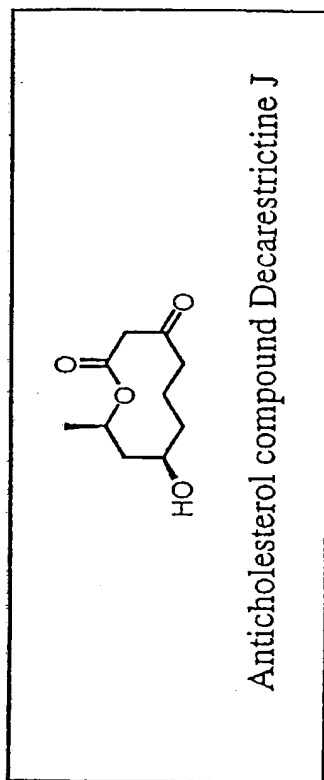


Figure 20.



**DECLARATION, PETITION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

(Check one):

- ☐ Declaration Submitted with Initial Filing
☒ Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**DNA MANIPULATION METHODS AND APPLICATIONS FOR
SYNTHETIC ENZYMES**

the specification of which (check one):

☐ is attached hereto.

OR

☒ was filed on 12 June 2000 as PCT International Application Number
PCT/GB00/02286 and filed as 9913694.7.

☐ and was amended by PCT Article 19 Amendment on _____
(if applicable),

☐ and was amended by PCT Article 34 Amendment on _____
(if applicable).

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

PRIORITY CLAIM

(Check one):

- ☐ no such applications have been filed.
- ☒ such applications have been filed as follows

1) **FOREIGN PRIORITY CLAIM:** I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (dd,mm,yyyy)	Priority Not Claimed	Certified Copy Attached	
				Yes	No
9913694.7	GB	11 June 1999 (11.06.99)	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

2) **PROVISIONAL PRIORITY CLAIM:** I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Provisional Application Number(s)	Filing Date (dd/mm/yyyy)

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

3) **U.S./PCT PRIORITY CLAIM:** I hereby claim the benefit under Title 35, United States Code, §120 of any United States application or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (dd/mm/yyyy)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

James E. Cockfield	Reg. No. <u>19,162</u>	Jeremiah Lynch	Reg. No. <u>17,425</u>
Thomas V. Smurzynski	Reg. No. <u>24,798</u>	David J. Ridders	Reg. No. <u>43,882</u>
Ralph A. Loren	Reg. No. <u>29,325</u>	Maria C. Laccotripe	Limited Recognition
Giulio A. DeConti, Jr.	Reg. No. <u>31,503</u>		Under 37 C.F.R. § 10.9(b)
Ann Lamport Hammitte	Reg. No. <u>34,858</u>	Debra J. Milasincic	Reg. No. <u>46,931</u>
Elizabeth A. Hanley	Reg. No. <u>33,505</u>	David R. Burns	Reg. No. <u>46,590</u>
Amy E. Mandragouras	Reg. No. <u>36,207</u>	Sean D. Detweiler	Reg. No. <u>42,482</u>
Anthony A. Laurentano	Reg. No. <u>38,220</u>	Cynthia L. Kanik	Reg. No. <u>37,320</u>
Kevin J. Canning	Reg. No. <u>35,470</u>	Theodore R. West	Reg. No. <u>47,202</u>
Jane E. Remillard	Reg. No. <u>38,872</u>	Shayne Y. Huff	Reg. No. <u>44,784</u>
DeAnn F. Smith	Reg. No. <u>36,683</u>	Hathaway P. Russell	Reg. No. <u>46,488</u>
Peter C. Lauro	Reg. No. <u>32,360</u>	Daniel B. Ko	Reg. No. <u>47,332</u>
Jeanne M. DiGiorgio	Reg. No. <u>41,710</u>	John S. Curran	Reg. No. <u>P50,445</u>
Megan E. Williams	Reg. No. <u>43,270</u>		

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Send Correspondence to:

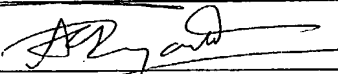
Anthony A. Laurentano, Esq., Lahive & Cockfield, LLP, 28 State Street, Boston, Massachusetts 02109, United States of America

Direct Telephone Calls to: (name and telephone number)

Anthony A. Laurentano, (617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Anand RANGANATHAN	
Inventor's signature 	Date 18/10/2002
Residence INDIA INX	
Citizenship India	
Post Office Address (if different) International Centre for Genetics Engineering and Biotechnology (ICGEB) Aruna Asaf Ali Marg, P.O. 10504, New Delhi 110067, Maharashtra, INDIA	

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